FORM PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C.371		**CCI-018US**
		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) **10/030850**
INTERNATIONAL APPLICATION **PCT/GB00/02662**	INTERNATIONAL FILING DATE **11 July 2000 (11.07.00)**	PRIORITY DATE CLAIMED **13 July 1999 (13.07.99)**

TITLE OF INVENTION
ORBIT AND HOMOLOGUES THEREOF

APPLICANT(S) FOR DO/EO/US
Maria do Carmo AVIDES, *et al.*

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C.371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **(unexecuted) (4 Sheets);**
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98;
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☒ A FIRST preliminary amendment **(8 Sheets);**
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: **PCT International Published Application (WO 01/04295 A1) (with International Search Report attached) (91 sheets); The International Preliminary Examination Report (7 sheets); Certificate of First Class Mailing (1 sheet); and Return Postcard.**

531 Rec'd PCT/PTO 11 JAN 2002

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NO.
10/030850	PCT/GB00/02662	CCI-018US

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) .(a/o November 1, 2000):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....**$1040**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.455(a)(2)) paid to USPTO **$740**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....**$710**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....**$100**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	**37**-20 =	**17**	X **$18.00**	$306.00	
Independent claims	**6**-3 =	**3**	X **$84.00**	$252.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **280.00**	$280.00	
TOTAL OF ABOVE CALCULATIONS =				$1858.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$1858.00	
SUBTOTAL =				$929.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$929.00	
				Amount to be: refunded	$
				charged	

a. ☐ Check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **12-0080** in the amount of $ **929.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **12-0080**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Giulio A. DeConti, Jr., Esq.
LAHIVE & COCKFIELD, LLP
28 State Street
Boston, Massachusetts 02109
United States of America
(617)227-7400
Date: 11 January 2002

SIGNATURE

Cynthia L. Kanik
NAME

37,320
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: **Maria do Carmo AVIDES, *et al.***

International Application No.: **PCT/GB00/02662**

International Filing Date: **July 11, 2000**

U.S. Serial No.: **010/030,850**

Filed: **January 11, 2002**

For: **ORBIT AND HOMOLOGUES THEREOF**

Attorney Docket No.: **CCI-018US**

Group Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

Commissioner for Patents
BOX PCT
Washington, D.C. 20231

TRANSMITTAL LETTER FOR DISKETTE CONTAINING SEQUENCE LISTING

Dear Sir:

Responsive to the Notification of Missing Requirements Under 35 U.S.C. §371 in the United States Designated/Elected Office (DO/EO/US) dated March 19, 2002, Applicants' attorney submits a substitute paper copy and a Computer Readable Form (diskette) of the Sequence Listing for the patent application filed on January 11, 2002. The substitute Sequence Listing complies with the requirements of 37 C.F.R. §1.821-1.825. The material on the enclosed diskette is identical in substance to the paper copy of the substitute Sequence Listing appearing on pages 1-13 submitted herewith. The computer readable form of the Sequence Listing contained on the enclosed diskette is understood to comply with the requirements of §1.824(d). The additional sequences listed in the substitute Sequence Listing contains sequences disclosed in the application, *i.e.*, at Figure 8, as filed on January 11, 2002. Accordingly, no new matter has been added

Immediately after the last figure, Figure 10, please substitute the hard (paper) copy of the substitute Sequence Listing, submitted herewith, in place of pages 1-6 of the Sequence Listing filed with the application on January 11, 2002.

"Express Mail" mailing label number EL 931 680 311 US

Date of Deposit October 18, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner for Patents, Box PCT, Washington, DC 20231

Viviato 6 Cardoso

Signature

Viviato 6 Cardoso

Please Print Name of Person Signing

Respectfully submitted,

LAHIVE & COCKFIELD, LLP

Cynthia L. Kanik, Ph.D.
Registration No. 37,320
Attorney for Applicants
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(617) 227-7400

(Atty. Docket No.: CCI-018US)

IN THE UNITED STATES PATENT DESIGNATED OFFICE (DO/US)
(National Phase of International Appln.: PCT/GB00/02662,
Publication No. WO 01/04295 A1)

In re the application of: **Maria do Carmo AVIDES, *et al.***

International Application No.: **PCT/GB00/02662**

International Filing Date: **11 July 2000**

U.S. Serial No.: **Not yet assigned**

Filed: **Herewith**

For: **ORBIT AND HOMOLOGUES THEREOF**

Attorney Docket No.: **CCI-018US**

Commissioner for Patents
BOX PCT
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Preliminary to the examination of the above-referenced patent application, please amend the application as follows.

In the Claims:

Please cancel claims 18 and 19 and amend claims 4, 5, 10, 11, 14, 15, 17, 21, 23 and 24 as follows:

4. (Amended) A polynucleotide probe which comprises a fragment of at least 15 nucleotides of the polynucleotide of claim 1.

5. (Amended) A polypeptide which comprises the sequence set out in SEQ ID No. 2 or a homologue, variant, derivative or fragment thereof.

10. (Amended) A method for detecting the presence or absence of a polynucleotide as defined in any one of claims 1 to 3 or 6 in a biological sample which comprises:

(a) bringing the biological sample containing DNA or RNA into contact with a probe which comprises a fragment of at least 15 nucleotides of the polynucleotide under hybridising conditions; and

(b) detecting any duplex formed between the probe and nucleic acid in the sample.

11. (Amended) A method for detecting a polypeptide which comprises the sequence set out in SEQ ID No. 2 or a fragment thereof present in a biological sample which comprises:

(a) providing an antibody capable of binding to the polypeptide or fragment thereof;

(b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said antibody is formed.

14. (Amended) An antibody according to claim 9 for use in therapy.

15. (Amended) A method of treating a tumour comprising administering to a patient in need of treatment an effective amount of a polynucleotide as defined in any one of claims 1 to 3 or 6.

17. (Amended) A method of treating a tumour, comprising administering to a patient in need of treatment an effective amount of an antibody as defined in claim 9.

21. (Amended) A substance identified by the method of claim 20.

23. (Amended) A process comprising the steps of:

(a) performing the method according claim 20; and

(b) preparing a quantity of those one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

24. (Amended) A process comprising the steps of:

(a) performing the method according claim 20; and

(b) preparing a pharmaceutical composition comprising one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

REMARKS

No new matter has been added. Claims 4, 10, 11, 14, 15, 17, 21, 23 and 24 were amended to correct obvious grammatical errors. Support for the amended claims can be found in the claims as originally filed.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP

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Date: **January 11, 2002**

APPENDIX A

Version with Markings to Show Changes Made

4. A polynucleotide probe which comprises a fragment of at least 15 nucleotides of the ~~a~~ polynucleotide ~~as defined in any one of~~ claim~~s~~ 1 ~~to 3~~.

5. A polypeptide which comprises the sequence set out in SEQ ID No. 2 or a homologue, variant, derivative or fragment thereof.

10. A method for detecting the presence or absence of a polynucleotide as defined in any one of claims 1 to 3 or 6 in a biological sample which comprises:

(a) bringing the biological sample containing DNA or RNA into contact with a probe which comprises a fragment of at least 15 nucleotides of the polynucleotide ~~according to claim 4~~ under hybridising conditions; and

(b) detecting any duplex formed between the probe and nucleic acid in the sample.

11. A method for detecting a polypeptide which comprises the sequence set out in SEQ ID No. 2 or a fragment thereof ~~as defined in claims 5~~ present in a biological sample which comprises:

(a) providing an antibody capable of binding to the polypeptide or fragment thereof ~~according to claim 9~~;

(b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said antibody is formed.

14. An antibody according to claim 9 ~~10~~ for use in therapy.

15. A method of treating a tumour comprising administering to a patient in need of treatment an effective amount of a polynucleotide as defined in any one of claims 1 to 3 or 6.

17. A method of treating a tumour, comprising administering to a patient in need of treatment an effective amount of an antibody as defined in claim 9 ~~10 to a patient~~.

21. A substance identified by the method of claim ~~18, 19 or~~ 20.

23. A process comprising the steps of:

(b) performing the method according claim ~~18, 19 or~~ 20; and

(b) preparing a quantity of those one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

24. A process comprising the steps of:

(d) performing the method according claim ~~18, 19 or~~ 20; and

(b) preparing a pharmaceutical composition comprising one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

APPENDIX B

Pending Claims

1. A polynucleotide encoding orbit or a homologue thereof.

2. A polynucleotide according to claim 1 wherein said homologue is human orbit.

3. A polynucleotide selected from:

(a) polynucleotides comprising a nucleotide sequence set out in SEQ ID No. 1 or the complement thereof.

(b) Polynucleotides comprising a nucleotide sequence capable of hybridising to the complement of the nucleotide sequence set out in SEQ ID No. 1 or a fragment thereof.

4. A polynucleotide probe which comprises a fragment of at least 15 nucleotides of the polynucleotide of claim 1.

5. A polypeptide which comprises the sequence set out in SEQ ID No. 2 or a homologue, variant, derivative or fragment thereof.

6. A polynucleotide encoding a polypeptide according to claim 5.

7. A vector comprising a polynucleotide as defined in any one of claims 1 to 3 or 6.

8. An expression vector comprising a polynucleotide as defined in any one of claims 1 to 3 or 6, operably linked to a regulatory sequence capable of directing expression of said polynucleotide in a host cell.

9. An antibody capable of binding the polypeptide of SEQ ID. No. 2 or a fragment thereof.

10. A method for detecting the presence or absence of a polynucleotide as defined in any one of claims 1 to 3 or 6 in a biological sample which comprises:

(a) bringing the biological sample containing DNA or RNA into contact with a probe which comprises a fragment of at least 15 nucleotides of the polynucleotide under hybridising conditions; and

(b) detecting any duplex formed between the probe and nucleic acid in the sample.

11. A method for detecting a polypeptide which comprises the sequence set out in SEQ ID No. 2 or a fragment thereof present in a biological sample which comprises:

(a) providing an antibody capable of binding to the polypeptide or fragment thereof;

(b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said antibody is formed.

12. A polynucleotide according to any one of claims 1 to 3 or 6 for use in therapy.

13. A polypeptide according to claim 5 for use in therapy.

14. An antibody according to claim 9 for use in therapy.

15. A method of treating a tumour comprising administering to a patient in need of treatment an effective amount of a polynucleotide as defined in any one of claims 1 to 3 or 6.

16. A method of treating a tumour, comprising administering to a patient in need of treatment an effective amount of a polypeptide as defined in claim 5.

17. A method of treating a tumour, comprising administering to a patient in need of treatment an effective amount of an antibody as defined in claim 9.

~~18. Use of an orbit polypeptide or homologue, derivative, variant or fragment thereof in a method of identifying a substance capable of affecting orbit function.~~

~~19. Use of an orbit polypeptide or homologue thereof, or fragment thereof in an assay for identifying a substance capable of inhibiting mitosis.~~

20. A method for identifying a substance capable of binding to an orbit polypeptide or a homologue, derivative, variant or fragment thereof, which method comprises incubating the orbit polypeptide or homologue, derivative, variant or fragment thereof with a candidate substance under suitable conditions and determining whether the substance binds the orbit polypeptide or homologue, derivative, variant or fragment thereof.

21. A substance identified by the method of claim 20.

22. A substance according to claim 21 for use in a method of inhibiting mitosis.

23. A process comprising the steps of:

(a) performing the method according claim 20; and

(b) preparing a quantity of those one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

24. A process comprising the steps of:

(a) performing the method according claim 20; and

(b) preparing a pharmaceutical composition comprising one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

ORBIT AND HOMOLOGUES THEREOF

Field of the Invention

The present invention relates to orbit, a *Drosophila* protein required for chromosome segregation, and homologues of orbit. The present invention also relates to the use of orbit and homologues thereof in assays to identify substances capable of inhibiting mitosis.

Background to the Invention

The requirement for microtubules in the mitotic spindle is self-evident, and yet the role of non-neuronal microtubule-associated proteins (MAPs) in its function is poorly understood. Formation of the spindle requires that the interphase microtubule network is reorganized as a result of an increase in microtubule turnover at mitotic entry. This is possible due to the dynamic instability of microtubules that oscillate between periods of growth and shrinkage and is due to an increase in the frequency of transitions from the polymerisation to depolymerisation phases, the frequency of catastrophe (for review, see Desai and Mitchison, 1997). How the catastrophe rate increases at the onset of mitosis has been puzzling as most known MAPs have the property of stabilizing microtubules. However, recent studies have identified proteins that promote microtubule catastrophe, such as op18 or stathmin, the microtubule-severing ATPase katanin, and the kin I family of kinesins (Belmont and Mitchison, 1996; Hartman *et al.*, 1998; Desai *et al.*, 1999).

Studies on the mitotic roles of MAPs have concentrated upon the use of *Xenopus* as a model system as it offers advantages for experimentation *in vitro*. Several MAPs have been identified that localize to the mitotic spindle including XMAP230, MAP4, XMAP215, and XMAP310 (Gard and Kirschner, 1987; Andersen, *et al.*, 1994; Vasquez, *et al.*, 1994; Ookata, *et al.*, 1995; Andersen and Karsenti, 1997; Charrasse, *et al.*, 1998). Their identification offers the possibility of direct studies of their effects upon microtubule dynamics.

We have chosen to search for mitotic regulators in *Drosophila melanogaster* which offers the possibility of studying the effects of mitotic mutations within the intact cell. The characterization of maternal effect mutants of *Drosophila* is a powerful route towards the identification of such genes. In many cases, maternal effect mitotic defects reflect a specific requirement for the product of the affected gene for cell division throughout development. The proteins encoded by such genes may have either regulatory roles or be part of the structural components of the mitotic apparatus. The protein kinases encoded by the *polo* and *aurora* genes, for example, were first identified through hypomorphic mutations which when homozygous in the mother result in gross mitotic defects within the embryo (Sunkel and Glover, 1988; Glover, *et al.*, 1995). However, the functions of these kinases in cell division throughout development has been revealed through the study of series of mutant alleles that show developmental arrest at different stages. A number of maternal effect mutations affecting the cytoskeletal organization of syncytial embryos have been identified. One of these, *nuclear fallout* (*nuf*), encodes a protein that concentrates at the centrosomes during prophase and is cytoplasmic during the rest of the nuclear cycle (Rothwell *et al.*, 1998). However, none of the genes originally identified by maternal effect mutations has yet been shown to encode a MAP, although the potential for identifying such mutants is evident. In fact, some alleles of *asp*, which encodes a protein associated with the polar regions of the mitotic spindle, exhibit a maternal effect on syncytial mitoses (Gonzalez *et al.*, 1990; Saunders *et al.*, 1997).

Alternative biochemical approaches to identify *Drosophila* MAPs also take advantage of the maternal dowry of proteins essential for the syncytial mitoses. One strategy has been to use libraries of monoclonal antibodies to search for proteins that display dynamic patterns of localization during the mitotic cycle (Frasch *et al.*, 1986). This set of antibodies was successful in identifying genes encoding a *Drosophila* homologue of the vertebrate regulator of chromatin condensation (RCC1) (Frasch, 1991), and a centrosomal antigen now known as CP190 (Whitfield *et al.*, 1988). Another strategy was to purify molecules based upon their ability to bind actin (Miller *et al.*, 1989) or microtubules (Kellogg *et al.*, 1989), and then raise antibodies against individual proteins. Some 50 proteins were identified that would bind to microtubules and monoclonal antibodies raised to 24 of them. One of the first to be cloned proved also to be CP190, which in turn was used as an affinity

reagent to identify a second centrosomal associated antigen CP60 (Kellogg and Alberts, 1992). The function of the majority of these proteins still remains uncertain because of the lack of mutations or assays of their molecular function.

We have continued the direct genetic approach in a search for mutants that identify genes encoding MAPs, expecting that these would be give rise to spindle defects in the syncytial mitoses, and also show defective cell divisions at other developmental stages. In this paper we report the characterization of one such novel gene *orbit*. We show that *orbit* encodes a novel 165 kDa MAP and discuss possible functions for this protein suggested by the phenotypes of an allelic series of *orbit* mutants.

Summary of the Invention

We have now identified a novel protein in *Drosophila*, termed orbit, and isolated genomic and cDNA clones encoding orbit. Orbit protein localises to mitotic spindles and binds microtubules. We have characterised several maternal-effect orbit mutant phenotypes which result in embryos exhibiting abnormal mitosis and polyploid cells.

Accordingly, the invention provides an orbit polypeptide or a homologue thereof. Typically, the orbit polypeptide or homologue thereof is a member of the class of proteins termed microtubule associated proteins (MAPs). The polypeptide preferably has one or more of the additional features:

(1) an HR1 domain having homology to amino acids 290 to 1093 of SEQ ID No. 2;

(2) an HR2 domain having homology to amino acids 1093 to 1271 of SEQ ID No. 2;

(3) at least one putative GTP binding domain, preferably one domain having substantial homology to amino acids 544 to 549 of SEQ ID No. 2 (GGGTGTG) and one domain having substantial homology to amino acids 400 to 403 of SEQ ID. No. 2 (NKLD);

(4) a molecular mass of from 150 to 190 kDa, as determined by SDS-PAGE;

(5) a highly basic domain (pI of at least 10.0 over at least 200, 250 or 300 amino acids), preferably flanked by short acidic domains (pI lower than 5.0) having less than 50 or 100 amino acids. Preferably said basic domain is homologous to amino acid residues 342 to 752 of SEQ I.D. No.2.

(6) at least one consensus $p34^{cdc2}$ phosphorylation site;

(7) binds to microtubules, preferably in a GTP-dependent manner;

(8) localises to the mitotic spindle.

Preferably, the degree of homology referred to in items (1), (2) and (5) is at least 15%, preferably at least 20, 25 or 30% amino acid identity. Preferably, the degree of substantial homology referred to in item (3) is at least 15%, preferably at least 50, 70 or 85% amino acid identity. Calculation of homology is described below.

Preferably the polypeptide is encoded by a cDNA sequence obtainable from a eukaryotic cDNA library, preferably a metazoan cDNA library (such as insect or mammalian) said DNA sequence comprising a DNA sequence being selectively detectable with a *Drosophila* orbit nucleotide sequence as shown in SEQ ID No. 1 or a fragment thereof.

The term "selectively detectable" means that the cDNA used as a probe is used under conditions where a target cDNA of the invention is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other cDNAs present in the cDNA library. In this event background implies a level of signal generated by interaction between the probe and a non-specific cDNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target cDNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P. Suitable conditions may be found by reference to the Examples.

The invention also provides the orbit protein of SEQ ID. 2 and derivatives, variants and homologues thereof, polypeptide fragments thereof, as well as antibodies capable of binding the orbit protein or polypeptide fragments thereof.

In another aspect, the present invention provides a polynucleotide selected from:

(a) polynucleotides comprising the nucleotide sequence set out in SEQ ID No. 1 or the complement thereof.

(b) polynucleotides comprising a nucleotide sequence capable of hybridising to the nucleotide sequence set out in SEQ ID No. 1, or a fragment thereof.

(c) polynucleotides comprising a nucleotide sequence capable of hybridising to the complement of the nucleotide sequence set out in SEQ ID No. 1 or a fragment thereof.

(d) polynucleotides comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the polynucleotides defined in (a), (b) or (c).

Also provided are polynucleotides encoding polypeptides of the invention. All such polynucleotides will be referred to as a polynucleotide of the invention. A polynucleotide of the invention includes a polynucleotide having a sequence as shown in SEQ ID No. 1 and fragments thereof capable of selectively hybridising to the *orbit* gene.

In a further aspect, the invention provides recombinant vectors carrying a polynucleotide of the invention, including expression vectors, and methods of growing such vectors in a suitable host cell, for example under conditions in which expression of a protein or polypeptide encoded by a sequence of the invention occurs.

In an additional aspect, the invention provides kits comprising polynucleotides, polypeptides or antibodies of the invention and methods of using such kits in diagnosing the presence of absence of orbit and their homologues, or variants thereof, including deleterious mutants.

In a further aspect, the present invention provides the use of an orbit polypeptide or homologue, derivative, variant or fragment thereof in a method of identifying a substance capable of affecting orbit function. For example, the invention provides the use of an orbit polypeptide or homologue, derivative, variant or fragment thereof in an assay for identifying a substance capable of inhibiting mitosis. Other possible orbit functions for which it may be desired to identify substances which affect such functions include microtubule binding, microtubule organising centre nucleation activity and interactions with microtubule motor proteins.

In this respect, the invention also provides a method for identifying a substance capable of binding to an orbit polypeptide or a homologue, derivative, variant or fragment thereof, which method comprises incubating the orbit polypeptide or homologue, derivative, variant or fragment thereof with a candidate substance and determining whether the substance binds to the orbit polypeptide or homologue, derivative, variant or fragment thereof.

Also provided is a substance identified by the above methods of the invention. Such substances may be used in a method of therapy, such as in a method of affecting orbit function, such as inhibiting mitosis.

The invention also provides a process comprising the steps of:

(a) performing one of the above methods; and

(b) preparing a quantity of those one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

Also provided is a process comprising the steps of:

(a) performing one of the above method; and

(b) preparing a pharmaceutical composition comprising one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

The present invention further provides a method of treating a tumour comprising administering to a patient in need of treatment an effective amount of a polynucleotide, polypeptide or antibody of the invention.

Detailed description of the invention.

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989)

and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

A. Polypeptides

It will be understood that polypeptides of the invention are not limited to polypeptides having the amino acid sequence set out in SEQ. ID. No. 2 or fragments thereof but also include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof.

Thus polypeptides of the invention also include those encoding orbit homologues from other species including animals such as mammals (e.g. mice, rats or rabbits), especially primates, more especially humans. More specifically, Orbit homologues included within the scope of the invention include human orbit.

Thus, the present invention covers variants, homologues or derivatives of the amino acid sequence set out in SEQ ID No. 2 of the present invention, as well as variants, homologues or derivatives of the nucleotide sequence coding for the amino acid sequences of the present invention.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 15, 20, 25, 30, 40, 50, 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least 50 or 100, preferably 200, 300, 400 or 500 amino acids with SEQ ID No. 2. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for protein function rather than non-essential neighbouring sequences. This is especially important when considering homologous sequences from distantly related organisms. Details of particular comparisons between *Drosophila* orbit and two human homologues are given in the Examples and indicate that amino acid homology (identity) can be as low as about 30% over the complete sequence or a fragment comprising the HR1

and/or HR2 domains. Homology may also be considered with respect to the homologous human sequences described below.

Particularly preferred regions over which to conduct homology comparisons are amino acids 290 to 1068 (HR1 domain) and/or 1093 to 1271 (HR2 domain) of SEQ ID. No. 2. Another important region is the basic region within the HR1 domain (amino acids 342 to 752 of SEQ I.D. No.2).

Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see http://www.ncbi.nih.gov/BLAST/), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410; FASTA is available for online searching at, for example, http://www.2.ebi.ac.uk.fasta3) and the GENEWORKS suite of comparison tools. However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence retains substantially the same activity as the unmodified sequence, preferably having at least the same activity as the polypeptides presented in the sequence listings.

Polypeptides having the amino acid sequence shown in SEQ I.D. No. 2, or fragments or homologues thereof may be modified for use in the present invention. Typically, modifications are made that maintain the biological activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified sequence retains the biological activity of the unmodified sequence. Alternatively, modifications may be made to deliberately inactivate one or more functional domains of the polypeptides of the invention. Amino acid substitutions may include the use of non-naturally occurring analogues, for example to increase blood plasma half-life of a therapeutically administered polypeptide.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention also include fragments of the full length sequences mentioned above. Preferably said fragments comprise at least one epitope. Fragments will typically comprise at least 6 amino acids, more preferably at least 10, 20, 30, 50 or 100 amino acids. Preferred fragments comprise functional domains of the full length orbit polypeptide (such as the HR1 and/or HR2 domains described above), for example fragments capable of binding to microtubules. In particular, preferred fragments comprise the basic domain.

In a particularly preferred aspect of the invention, the full length human KIAA0622 and KIAA0627 proteins described amongst a list of cDNA clones of unknown function provided by Ishikawa *et al.*, 1998, are specifically excluded from the scope of the term "polypeptides of the invention". The full length sequences of these proteins is available under Accession nos. BAA31597 and BAA31602. However, it is preferred that orbit polypeptide homologues within the scope of the invention include polypeptides having less than 99, 98, 95 or 90% homology but more than 30, 40 or 50% homology to the full length sequences set out as Accession nos. BAA31597 and BAA31602.

Proteins of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Proteins of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and

the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the function of the protein of interest sequence.

Proteins of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A protein of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a protein of the invention.

A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled polypeptides of the invention may be used in diagnostic procedures such as immunoassays to determine the amount of a polypeptide of the invention in a sample. Polypeptides or labelled polypeptides of the invention may also be used in serological or cell-mediated immune assays for the detection of immune reactivity to said polypeptides in animals and humans using standard protocols.

A polypeptide or labelled polypeptide of the invention or fragment thereof may also be fixed to a solid phase, for example the surface of an immunoassay well or dipstick. Such labelled and/or immobilised polypeptides may be packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like. Such polypeptides and kits may be used in methods of detection of antibodies to the orbit polypeptides or their allelic or species variants by immunoassay.

Immunoassay methods are well known in the art and will generally comprise:

(a) providing a polypeptide comprising an epitope bindable by an antibody against said protein;

(b) incubating a biological sample with said polypeptide under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said polypeptide is formed.

Polypeptides of the invention may be used in *in vitro* or *in vivo* cell culture systems to study the role of orbit and its homologues in disease. For example, truncated or modified orbit may be introduced into a cell to disrupt the normal functions which occur in the cell. Specific examples may include fragments of orbit or its homologues which comprise only the basic domain (within the HR1 domain). The polypeptides of the invention may be introduced into the cell by *in situ* expression of the polypeptide from a recombinant expression vector (see below). The expression vector optionally carries an inducible promoter to control the expression of the polypeptide.

The use of mammalian host cells is expected to provide for such post-translational modifications (e.g. myristolation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Such cell culture systems in which polypeptides of the invention are expressed may be used in assay systems to identify candidate substances which interfere with or enhance the functions of the polypeptides of the invention in the cell.

B. Polynucleotides

Polynucleotides of the invention include polynucleotides comprises the nucleic acid sequence set out in SEQ ID No. 1 and fragments thereof. Polynucleotides of the invention also include polynucleotides encoding the polypeptides of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed.

Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

The terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence of the present invention include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence. Preferably said variant, homologues or derivatives code for a polypeptide having biological activity, such as microtubule binding activity, preferably having substantially the same activity as the amino acid sequence shown as SEQ ID No. 2.

In a particularly preferred aspect of the invention, the full length human KIAA0622 and KIAA0627 nucleotide sequences described amongst a list of cDNA clones of unknown function provided by Ishikawa *et al.*, 1998, are specifically excluded from the scope of the term "polynucleotides of the invention". The full length sequences of these nucleotides is available under Accession nos. BAA31597 and BAA31602. However, it is preferred that homologues within the scope of the invention include nucleotides having less than 99, 98, 95 or 90% homology but more than 30, 40 or 50% homology to the full length sequences set out as Accession nos. BAA31597 and BAA31602.

As indicated above, with respect to sequence homology, preferably there is at least 50 or 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical

nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length.

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction technologies.

Polynucleotides of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred polynucleotides of the invention will comprise regions encoding polypeptide domains homologous to the polypeptide domains described above (for example the HR1, HR2 or basic domains), preferably at least 70, 80 or 90% and more preferably at least 95% homologous to said regions.

The term "selectively hybridizable" means that the polynucleotide used as a probe is used under conditions where a target polynucleotide of the invention is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other polynucleotides present, for example, in the cDNA or genomic DNA library being screening. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0).

Where the polynucleotide of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also included within the scope of the present invention.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of SEQ I.D. No 1 under conditions of

medium to high stringency. More preferably, the nucleotide sequences of the human KIAA0622 and/or KIAA0627 proteins described by Ishikawa *et al.*, 1998 (Accession nos. BAA31597 and BAA31602), or fragments thereof, may be used to identify other primate/mammalian homologues since nucleotide homology between human sequences and mammalian sequences is likely to be higher than is the case for the *Drosophila* sequence identified herein.

Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences. It will be appreciated by the skilled person that overall nucleotide homology between sequences from distantly related organisms is likely to be very low and thus in these situations degenerate PCR may be the method of choice rather than screening libraries with labelled fragments of SEQ I.D. No. 1.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences, such as SEQ ID. No 1. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides. For example, further changes may be desirable to represent particular coding changes found in orbit which give rise to

mutant orbit genes which have lost their regulatory function. Probes based on such changes can be used as diagnostic probes to detect such orbit mutants.

Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as a DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector

Polynucleotides or primers of the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the invention and may be detected using by techniques known *per se*.

Polynucleotides or primers of the invention or fragments thereof labelled or unlabelled may be used by a person skilled in the art in nucleic acid-based tests for detecting or sequencing orbit and its homologues in the human or animal body.

Such tests for detecting generally comprise bringing a biological sample containing DNA or RNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridising conditions and detecting any duplex formed between the probe and nucleic acid in the sample. Such detection may be achieved using techniques such as PCR or by immobilising the probe on a solid support, removing nucleic acid in the sample which is not hybridised to the probe, and then detecting nucleic acid which has hybridised to the probe. Alternatively, the sample nucleic acid may be immobilised on a solid support, and the amount of probe bound to such a support can be detected. Suitable assay methods of this and other formats can be found in for example WO89/03891 and WO90/13667.

Tests for sequencing orbit and its homologues include bringing a biological sample containing target DNA or RNA into contact with a probe comprising a polynucleotide or primer of the invention under hybridising conditions and determining the sequence by, for example the Sanger dideoxy chain termination method (see Sambrook *et al.*).

Such a method generally comprises elongating, in the presence of suitable reagents, the primer by synthesis of a strand complementary to the target DNA or RNA and selectively terminating the elongation reaction at one or more of an A, C, G or T/U residue; allowing strand elongation and termination reaction to occur; separating out according to size the elongated products to determine the sequence of the nucleotides at which selective termination has occurred. Suitable reagents include a DNA polymerase enzyme, the deoxynucleotides dATP, dCTP, dGTP and dTTP, a buffer and ATP. Dideoxynucleotides are used for selective termination.

Tests for detecting or sequencing orbit, or its homologue, in a biological sample may be used to determine orbit sequences within cells in individuals who have, or are suspected to have, an altered orbit gene sequence, for example within cancer cells including leukaemia cells and

solid tumours such as breast, ovary, lung, colon, pancreas, testes, liver, brain, muscle and bone tumours.

In addition, the discovery of orbit will allow the role of this gene in hereditary diseases to be investigated. In general, this will involve establishing the status of orbit, or its homologue (e.g. using PCR sequence analysis), in cells derived from animals or humans with, for example, neurological disorders or neoplasms.

The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may also contain suitable reagents for treating the sample to be probed, hybridising the probe to nucleic acid in the sample, control reagents, instructions, and the like.

C. Nucleic acid vectors

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

Vectors of the invention may be transformed or transfected into a suitable host cell as described below to provide for expression of a protein of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the protein, and optionally recovering the expressed protein. Vectors will be chosen that are compatible with the host cell used.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

Control sequences operably linked to sequences encoding the polypeptide of the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term promoter is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in mammalian cells, although prokaryotic promoters and promoters functional in other eukaryotic cells, such as insect cells, may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of α-actin,

β-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

Polynucleotides according to the invention may also be inserted into the vectors described above in an antisense orientation to provide for the production of antisense RNA. Antisense RNA or other antisense polynucleotides may also be produced by synthetic means. Such antisense polynucleotides may be used in a method of controlling the levels of orbit or its variants or species homologues.

D. Host cells

Vectors and polynucleotides of the invention may be introduced into host cells for the purpose of replicating the vectors/polynucleotides and/or expressing the polypeptides of the invention encoded by the polynucleotides of the invention. Although the polypeptides of the invention may be produced using prokaryotic cells as host cells, it is preferred to use eukaryotic cells, for example yeast, insect or mammalian cells, in particular mammalian cells.

Vectors/polynucleotides of the invention may be introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and

electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

E. Protein Expression and Purification

Host cells comprising polynucleotides of the invention may be used to express polypeptides of the invention. Host cells may be cultured under suitable conditions which allow expression of the proteins of the invention. Expression of the polypeptides of the invention may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG.

Polypeptides of the invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption.

Polypeptides of the invention may also be produced recombinantly in an *in vitro* cell-free system, such as the TnT™ (Promega) rabbit reticulocyte system.

F. Antibodies.

The invention also provides monoclonal or polyclonal antibodies to polypeptides of the invention or fragments thereof. Thus, the present invention further provides a process for the production of monoclonal or polyclonal antibodies to polypeptides of the invention.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing an orbit epitope(s). Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an orbit epitope contains antibodies to other

antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against orbit epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

Antibodies, both monoclonal and polyclonal, which are directed against orbit epitopes are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the agent against which protection is desired.

Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful in therapy.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv). Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400.

Antibodies may be used in method of detecting polypeptides of the invention present in biological samples by a method which comprises:

(a) providing an antibody of the invention;

(b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said antibody is formed.

Suitable samples include extracts tissues such as brain, breast, ovary, lung, colon, pancreas, testes, liver, muscle and bone tissues or from neoplastic growths derived from such tissues.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

G. Assays

The present invention provides assay that are suitable for identifying substances that bind to orbit polypeptides (reference to which includes homologues, variants, derivatives and fragments as described above). In addition, assays are provided that are suitable for identifying substances that interfere with orbit binding to components of the mitotic/meiotic machinery, in particular microtubules. Such assays are typically *in vitro*. Assays are also provided that test the effects of candidate substances identified in preliminary *in vitro* assays on intact cells in whole cell assays.

Candidate substances

A substance that inhibits cell division (including mitosis and/or meiosis) as a result of an interaction with orbit polypeptides may do so in several ways. It may directly disrupt the binding of orbit to a component of the spindle apparatus by, for example, binding to orbit and masking or altering the site of interaction with the other component. Candidate substances of this type may conveniently be preliminarily screened by *in vitro* binding

assays as, for example, described below and then tested, for example in a whole cell assay as described below. Examples of candidate substances include antibodies which recognise orbit.

A substance which can bind directly to orbit may also inhibit its function in cell division by altering its subcellular localisation thus preventing orbit and components of the mitotic apparatus from coming into contact within the cell. This can be tested using, for example the whole cells assays described below. Non-functional homologues of orbit may also be tested for inhibition of mitosis since they may compete with orbit for binding to components of the mitotic apparatus whilst being incapable of the normal functions of orbit or block the function of orbit bound to the mitotic apparatus. Such non-functional homologues may include naturally occurring orbit mutants and modified orbit sequences or fragments thereof. In particular, fragments of orbit which comprise the basic domain but lack other functional domains may be used to compete with full length orbit for binding to microtubules.

Alternatively, instead of preventing the association of the components directly, the substance may suppress the biologically available amount of orbit. This may be by inhibiting expression of the component, for example at the level of transcription, transcript stability, translation or post-translational stability. An example of such a substance would be antisense RNA or double-stranded interfering RNA sequences which suppresses the amount of orbit mRNA biosynthesis.

Suitable candidate substances include peptides, especially of from about 5 to 30 or 10 to 25 amino acids in size, based on the sequence of the various domains of *Drosophila* orbit described in section A, or variants of such peptides in which one or more residues have been substituted. Peptides from panels of peptides comprising random sequences or sequences which have been varied consistently to provide a maximally diverse panel of peptides may be used.

Suitable candidate substances also include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted

antibodies) which are specific for orbit. Furthermore, combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as inhibitors of binding of orbit to the mitotic apparatus (such as microtubules). The candidate substances may be used in an initial screen in batches of, for example 10 substances per reaction, and the substances of those batches which show inhibition tested individually. Candidate substances which show activity in *in vitro* screens such as those described below can then be tested in whole cell systems, such as mammalian cells which will be exposed to the inhibitor and tested for inhibition of mitosis.

Orbit binding assays

One type of assay for identifying substances that bind to orbit involves contacting an orbit polypeptide, which is immobilised on a solid support, with a non-immobilised candidate substance determining whether and/or to what extent the orbit polypeptide and candidate substance bind to each other. Alternatively, the candidate substance may be immobilised and the orbit polypeptide non-immobilised.

In a preferred assay method, the orbit polypeptide is immobilised on beads such as agarose beads. Typically this is achieved by expressing the component as a GST-fusion protein in bacteria, yeast or higher eukaryotic cell lines and purifying the GST-fusion protein from crude cell extracts using glutathione-agarose beads (Smith and Johnson, 1988). As a control, binding of the candidate substance, which is not a GST-fusion protein, to the immobilised orbit polypeptide is determined in the absence of the orbit polypeptide. The binding of the candidate substance to the immobilised orbit polypeptide is then determined. This type of assay is known in the art as a GST pulldown assay. Again, the candidate substance may be immobilised and the orbit polypeptide non-immobilised.

It is also possible to perform this type of assay using different affinity purification systems for immobilising one of the components, for example Ni-NTA agarose and histidine-tagged components.

Binding of the orbit polypeptide to the candidate substance may be determined by a variety of methods well-known in the art. For example, the non-immobilised component may be labelled (with for example, a radioactive label, an epitope tag or an enzyme-antibody conjugate). Alternatively, binding may be determined by immunological detection techniques. For example, the reaction mixture can be Western blotted and the blot probed with an antibody that detects the non-immobilised component. ELISA techniques may also be used.

Candidate substances are typically added to a final concentration of from 1 to 1000 nmol/ml, more preferably from 1 to 100 nmol/ml. In the case of antibodies, the final concentration used is typically from 100 to 500 μg/ml, more preferably from 200 to 300 μg/ml.

Microtubule binding/polymerisation assays

Another type of *in vitro* assay involves determining whether a candidate substance modulates binding of orbit to microtubules. Such an assay typically comprises contacting orbit protein with microtubules in the presence or absence of the candidate substance and determining if the candidate substance has an affect on orbit binding. Microtubules may be prepared as described in the Examples. Suitable assays for measuring GTP-dependent binding of orbit to microtubules *are* also described in the Examples. It may be desirable in one embodiment of this type of assay to deplete orbit protein from cell extracts used to produce polymerise microtubules. This may, for example, be achieved by the use of anti-orbit antibodies.

A simple extension to this type of assay would be to test the effects of purified Orbit protein upon the ability of tubulin to polymerise *in vitro* (for example, as used by Andersen and Karsenti, 1997) in the presence or absence of a candidate substance (typically added at the concentrations described above). *Xenopus* cell-free extracts may conveniently be used, for example as a source of tubulin.

Microtubule organising centre (MTOC) nucleation activity assays

Candidate substances, for example those identified using the Orbit binding assays described above, may be screening using a microtubule organising centre nucleation activity assay to determine if they are capable of disrupting MTOCs as measured by, for example, aster formation. This assay in its simplest form comprises adding the candidate substance to a cellular extract which in the absence of the candidate substance has microtubule organising centre nucleation activity resulting in formation of asters.

In a preferred embodiment, the assay system comprises (i) an Orbit polypeptide or homologue, variant, derivative, fragment thereof and (ii) components required for microtubule organising centre nucleation activity except for functional Orbit, which is typically removed by immunodepletion (or by the use of extracts from Orbit mutants). The components themselves are typically in two parts such that microtubule nucleation does not occur until the two parts are mixed (microtubule nucleation can take place in the absence of Orbit but the result is a mass of disorganised microtubules rather than normal aster. The Orbit may be present in one of the two parts initially or added subsequently prior to mixing of the two parts.

Subsequently, the Orbit polypeptide and candidate substance are added to the component mix and microtubule nucleation from centrosomes measured, for example by immunostaining for Orbit and visualising aster formation by immuno-fluorescence microscopy. Orbit polypeptide may be preincubated with the candidate substance before addition to the component mix. Alternatively, both Orbit polypeptide and the candidate substance may be added directly to the component mix, simultaneously or sequentially in either order.

The components required for microtubule organising centre formation typically include salt-stripped centrosomes prepared as described in Moritz *et al.*, 1998. Stripping centrosome preparations with 2 M KI removes the centrosome proteins CP60, CP190, CNN and γ-tubulin. Of these, neither CP60 nor CP190 appear to be required for microtubule nucleation. The other minimal components are typically provided as an orbit-depleted cellular extract, or conveniently, as a cellular extract from cells with non-

functional orbit, for example *Drosophila* embryo extracts from orbit-mutant embryos. Typically, labelled tubulin (usually β-tubulin) is also added to assist in visualising aster formation.

Alternatively, partially purified centrosomes that have not been salt-stripped may be used as part of the components. In this case, only tubulin, preferably labelled tubulin is required to complete the component mix.

Candidate substances are typically added to a final concentration of from 1 to 1000 nmol/ml, more preferably from 1 to 100 nmol/ml. In the case of antibodies, the final concentration used is typically from 100 to 500 μg/ml, more preferably from 200 to 300 μg/ml.

The degree of inhibition of aster formation by the candidate substance may be determined by measuring the number of normal asters per unit area for control untreated cell preparation and measuring the number of normal asters per unit area for cells treated with the candidate substance and comparing the result. Typically, a candidate substance is considered to be capable of disrupting MTOC integrity if the treated cell preparations have less than 50%, preferably less than 40, 30, 20 or 10% of the number of asters found in untreated cells preparations. It may also be desirable to stain cells for γ-tubulin to determine the maximum number of possible MTOCs present to allow normalisation between samples.

Motor protein assay

The genetic interaction of *orbit* with *klp61F* indicates that the two proteins may interact directly and/or that Orbit influences the activity of the Eg5-like motor protein *in vitro*. The effects of candidate substances on such a process may be determined using assays wherein the motor protein is immobilised on coverslips. Rhodamine labelled microtubules are then added and their translocation can be followed by fluorescent microscopy. The effect of candidate substances may thus be determined by comparing the extent and/or rate of translocation in the presence and absence of the candidate substance. Generally, candidate

substances known to bind orbit, or non-functional Orbit protein, or a fragment or derivative thereof, would be tested in this assay. Alternatively, a high throughput assay may be used to identify modulators of motor proteins and the resulting identified substances tested for affects on orbit binding as described above.

Typically this assay uses microtubules stabilised by taxol (e.g. Howard and Hyman 1993; Chandra and Endow, 1993 – both chapters in "Motility Assays for Motor Proteins" Ed Jon Scholey, pub Academic Press). If however, Orbit protein were to promote stable polymerisation of microtubules (see above) then these microtubules could be used directly in motility assays.

Assay for spindle assembly and function

A further assay to investigate orbit function and the effect of candidate substances on orbit function is as assay which measures spindle assembly and function. Typically, such assays are performed using *Xenopus* cell free systems, where two types of spindle assembly are possible. In the "half spindle" assembly pathway, a cytoplasmic extract of CSF arrested oocytes is mixed with sperm chromatin. The half spindles that form subsequently fuse together. A more physiological method is to induce CSF arrested extracts to enter interphase by addition of calcium, whereupon the DNA replicates and kinetochores form. Addition of fresh CSF arrested extract then induces mitosis with centrosome duplication and spindle formation (for discussion of these systems see Tournebize and Heald, 1996).

Again, generally, candidate substances known to bind orbit, or non-functional Orbit protein, or a fragment or derivative thereof, would be tested in this assay. Alternatively, a high throughput assay may be used to identify modulators of spindle formation and function and the resulting identified substances tested for affects on orbit binding as described above.

Whole cell assays

Candidate substances may also be tested on whole cells for their effect on cell division, including mitosis and/or meiosis. Preferably the candidate substances have been identified by the above-described *in vitro* methods. Alternatively, rapid throughput screens for substances capable of inhibiting cell division, typically mitosis, may be used as a preliminary screen and then used in the *in vitro* assay described above to confirm that the affect is on orbit.

The candidate substance, i.e. the test compound, may be administered to the cell in several ways. For example, it may be added directly to the cell culture medium or injected into the cell. Alternatively, in the case of polypeptide candidate substances, the cell may be transfected with a nucleic acid construct which directs expression of the polypeptide in the cell. Preferably, the expression of the polypeptide is under the control of a regulatable promoter.

Typically, an assay to determine the effect of a candidate substance identified by the method of the invention on cell mitosis comprises administering the candidate substance to a cell and determining whether the substance inhibits mitosis. Techniques for measuring mitosis in a cell population are well known in the art. The extent of mitosis in treated cells is compared with the extent of mitosis in an untreated control cell population to determine the degree of inhibition, if any.

The concentration of candidate substances used will typically be such that the final concentration in the cells is similar to that described above for the *in vitro* assays.

A candidate substance is typically considered to be an inhibitor of mitosis if mitosis is reduced to below 50%, preferably below 40, 30, 20 or 10% of that observed in untreated control cell populations.

H. Therapeutic uses

Many tumours are associated with rapid and often aberrant mitosis. One therapeutic approach to treating cancer is to inhibit mitosis in rapidly dividing cells. Thus, since orbit appears to be required for the normal mitotic process, it represents a target for inhibition of cell division, particularly in tumour cells.

One possible approach is to express anti-sense orbit constructs, preferably selectively in tumour cells, to inhibit orbit function and prevent tumour cell division. Another approach is to use non-functional variants of orbit that compete with orbit for cellular components of mitosis, resulting in inhibition of mitosis. Alternatively, compounds identified by the assays described above as binding to orbit may be administered to tumour cells to prevent orbit function. This may be performed, for example, by means of gene therapy or by direct administration of the compounds. Anti-orbit antibodies may also be used as therapeutic agents.

I. Administration

Substances identified or identifiable by the assay methods of the invention may preferably be combined with various components to produce compositions of the invention. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition of the invention may be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

Polynucleotides/vectors encoding polypeptide components (or antisense constructs) for use in inhibiting mitosis may be administered directly as a naked nucleic acid construct. They may further comprise flanking sequences homologous to the host cell genome. When the

polynucleotides/vectors are administered as a naked nucleic acid, the amount of nucleic acid administered may typically be in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg. It is particularly preferred to use polynucleotides/ vectors that target specifically tumour cells, for example by virtue of suitable regulatory constructs or by the use of targeted viral vectors.

Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

Preferably the polynucleotide or vector according to the invention is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention. The Examples refer to the Figures. In the Figures:

Detailed Description of the Figures

Figure 1. The formation of polyploid nuclei and the failure of proper chromatid segregation were observed during nuclear division cycles in embryos derived from females

homozygous for *orbit*^{1}. Wild-type embryos (A, B) and *orbit*^{1} -derived embryos (C, D) at syncytial blastoderm stages were stained with Hoechst 33258 to visualize DNA.

(A) Wild-type embryos at interphase of cycle 13 show even distributions of nuclei of the same size.

(B) At anaphase of cycle 10 in the wild-type embryos, chromatids segregate toward both poles.

(C) Uneven distribution of nuclei at interphase in an *orbit*^{1} -derived syncytial blastoderm. In this field, there are at least three nuclei that appear to contain more than a diploid DNA content because of their larger size and the brighter staining with Hoechst (broad arrowheads). Pairs of sister nuclei which appear associated with each other possibly due to the incomplete separation of chromosomes or to nuclear fusion at telophase are indicated by narrow arrowheads. Additional arrows indicate pairs of sister nuclei connected together by chromatin bridges suggesting failure in chromosome segregation.

(D) *orbit*^{1} -derived embryos in anaphase of cycle 10. Arrowheads point to two nuclei which failed to undergo bi-polar segregation of chromatids. Bar, 10 mm.

Figure 2. Abnormal mitotic spindles in *orbit*^{1}-derived embryos. Wild-type embryos (A, B) and embryos derived from *orbit*^{1} females (C-F) were fixed and incubated with a rat anti-tubulin antibody, YL1/2 and then FITC-conjugated anti-rat IgG to visualize microtubules (green). With the exception of panel (F), the embryos were also incubated with Rb188, a rabbit antibody against the centrosome protein, CP190 and then with Cy5-conjugated anti-rabbit IgG to visualize centrosomes (blue). DNA was revealed by staining with propidium iodide (red).

Normal mitotic figures at metaphase (A) or at anaphase (B) during nuclear divisions in syncytial blastoderms.

(C) Free centrosomes not associated with chromosomes are evident in cytoplasm of the syncytial blastoderm embryos from *orbit*^{1}. Arrowheads indicate abnormal metaphase figures in which tetrapolar mitotic spindle formed between fully separated centrosomes at opposite poles and a free centrosome.

(D) The arrows indicate bent or curved mitotic spindles extended from incompletely separated centrosomes.

(E) There are many abnormal microtubule bundles which have a wavy structure. In anaphase figures associated with such abnormal spindles, some of chromosomes were left in the midzone between poles.

(F) Abnormal orientations of midbody microtubules at telophase. The arrowheads indicate telophase figures in which midbody microtubules failed to form with correct orientation to the sister nuclei. Bar in each panel, 10 mm.

Figure 3. Cytological phenotypes in squashed preparations of larval CNS cells from third instar larvae homozygous for *orbit*^{1} or from third instar larvae trans-heterozygous for *orbit*^{2} and *orbit*^{3}. Larval CNS cells stained with aceto-orcein were squashed and the mitotic figures were observed by phase-contrast microscopy.

(A) A diploid female metaphase figure and (B) a diploid anaphase figure from wild-type.

(C) A diploid metaphase figure containing hyper-condensed chromosomes from *orbit*^{1} homozygotes.

(D) A circular mitotic figure from *orbit*^{1} homozygous larvae. Note that all arms of major chromosomes are oriented to the periphery and the small dot-like fourth chromosomes are in the centre. (E) A monopolar anaphase-like figure in which all chromatids appear to be pulled toward a single pole.

(F) A polyploid mitotic figures from lethal mutant *orbit*^{2}/*orbit*^{3}, in which highly over-condensed chromatids or chromosomes were observed.

(G) A polyploid mitotic figure containing hyper-condensed chromosomes from an *orbit*^{1} homozygous larva.

(H) A lower magnification view of an extremely hyperploid mitotic figure from *orbit*^{2}/*orbit*^{3} larva. More than one hundred dot-like chromosomes were contained in this single mitotic cell. Bars, 10mm.

Figure 4. Immunostaining of centrosomes and mitotic spindles in whole-mount preparations of larval CNS cells from *orbit*^{1} homozygotes. (A-C) Centrosomes were detected by immunostaining using the rabbit antibody Rb188 followed by Texas Red-conjugated anti-rabbit IgG. Simultaneously chromosomes were also stained with propidium iodide. In left column and in the merged images presented in the right column, chromosomes and centrosomes are represented in red or orange. Microtubules were

stained with the anti-tubulin antibody, YL1/2 and subsequently with FITC-conjugated secondary antibody. In the middle column and the merged images in right column, mitotic spindles are shown in green. For (D), chromosomes stained with propidium iodide are represented in red in the right column and the merged image in left column. Centrosomes stained with Rb188 and then with FITC-conjugated secondary antibody are represented in green in middle column and the merged image.

(A) Normal bipolar spindles organized by fully separated centrosomes at opposite poles in normal metaphase cell.

(B) Polyploid cells containing multi-polar spindles. Arrowheads indicate positions on which spindle poles appear to be localized. Note that the third spindle pole is localized on a lower confocal plane than plane on which another two poles lay.

(C) Centrosomes appear yellow as a consequence of the overlapped staining by YL1/2 and Rb188 antibodies. Chromatids appear to be pulled toward the single spindle pole.

(D) A circular mitotic figure in which a single centrosome is situated in the centre of chromosomes and is localized on same confocal plane as chromosome are. Bars, 10 mm.

Figure 5. Cytological and physical maps of the *orbit* region.

(A) Polytene chromosome map of the 78C region within which the *P-lacW* element responsible for the *orbit*^{1} phenotypes was localized by *in situ* hybridization. The cytological extents of four deficiencies are shown as thick lines below the polytene chromosome map. Thin lines indicates an uncertainty in deficiency breakpoint ends. The *orbit* mutation was included in the *Df(3L)Pc-9a* and *Df(3L)Pc-12h* but not in *Df(3L)Pc-14d* nor *Df(3L)Pc*. Chromosome breakpoints were published by Russell *et al.*, 1996.

(B) Horizontal lines represent the genomic regions contained in two cosmid clones 52G10 and 76D10, which were isolated from a wild-type genomic library (Sieden-Kiamos, 1990) using DNA flanking the *P-lacW* in *orbit*^{1} that was retrieved by plasmid rescue.

(C) Restriction enzyme map of the *orbit* region. Cleavage sites are: E, *Eco*RI ; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I. The position of the *P-lacW* insertion is indicated relative to the genomic DNA. Its orientation is indicated by the *lacZ* gene within the transposon.

(D) Genomic extents of *orbit* and *asparagine synthetase* gene transcription units and their directions of transcription. The cDNA clones for two transcription units were isolated from 0-8 hour or 0-22 hour embryonic cDNA libraries or testis cDNA library using as

probes a mixture of the 1.3 kb and 1.0 kb long flanking DNA of the P element derived by plasmid rescue from both sides. The extent of each cDNA clone is presented below the transcription mapsd.

(E) Extent of genomic deletions associated with the three lethal alleles. The deletion associated with the *orbit*^{2} extends from the 0.9 kb *Bam*HI fragment in the *aspartate synthetase* gene to a position inside the P element. Therefore it can be estimated that the deletion includes the regulatory region of the *orbit* and part of the coding region of the *aspartate synthetase* gene. A deletion associated with the *orbit*^{4} includes a genomic region from within the 0.9 kb *Bam*HI fragment in the *aspartate synthetase* gene to the 2.0 kb *Eco*RI-*Hin*dIII fragment in the *orbit* gene. For *orbit*^{3}, a genomic region extending from 5' regulatory region of the *orbit* gene to the 2.0 kb *Eco*RI-*Hin*dIII fragment within the *orbit* gene was estimated to be deleted.

(F) The 14 kb *Bam*HI fragment from cosmid 52G10 was used to construct pB14 transformation plasmid. It extends from the *Bam*HI site at the 3' end region of the *aspartate synthetase* gene to a genomic *Bam*HI site at the 3' end of the cosmid 52G10.

Figure 6. Northern analysis of *orbit* mRNA and identification of the gene product on Western blot.

(A) Developmental expression of *orbit* in wild-type animals. (Lane 1) 0-2 hour embryos; (lane 2) 2-4 hour embryos; (lane 3) 4-8 hour embryos; (lane 4) 8-12 hours embryos; (lane 5) 12-16 hour embryos; (lane 6) 16-22 hour embryos; (lane 7) first instar larvae; (lane 8) second instar larvae; (lane 9) third instar larvae; (lane 10) pupae; (lane 11) adult males; (lane 12) adult females. 20 µg of total RNA were loaded in each lane. A 2 kb *Eco*RI fragment from a *orbit* cDNA clone was used as a probe. The same blot filter hybridized with *rp49* cDNA as a loading control.

(B) Expression of *orbit* in adult flies homozygous for *orbit*^{1}. (Lane 1) wild-type adult females; (lane 2) *orbit*^{1} */orbit*^{1} adult females; (lane 3) *orbit*^{1} */orbit*^{1} adult females; (lane 4) *orbit*^{1} */orbit*^{1} adult males; (lane 5) wild-type adult males. 20 µg of total RNA were loaded in each lane. The blot filter hybridized with same probes as in A.

(C) Identification of the Orbit polypeptide on Western blot and reduction in amount of the polypeptide of 165 kDa in protein extracts from the *orbit* mutants. (Lane 1) Protein extracts from three wild-type ovaries; (lane 2) protein extracts from ten *orbit*^{1} */orbit*^{1}

ovaries; (lane 3) protein extracts from ten wild-type larval brains; (lane 4) protein extracts from twenty *orbit*^{2}/*orbit*^{3} larval brains. An affinity-purified polyclonal antibody against a polypeptide corresponding to residues 1 to 654 recognized two bands, a major band of 165 kDa and a minor band of 90 kDa on Western blot. The major band is reduced in amount in the extracts from *orbit*^{1} ovaries and is barely detectable in the brain extracts from the lethal mutants, whereas a minor band shows no changes in amount. As the molecular weight of the major band is almost the same as the predicted molecular weight from the amino acid sequence, it is concluded that the major band of 165 kDa corresponds to the Orbit protein and that the minor band is not a proteolysis-product of the Orbit but cross-reaction of the antibody with another polypeptide. The detection of comparable amounts of the 90 kDa band between lanes indicates that equal amounts of proteins were blotted on each lane of the filter.

Figure 7. The amino acid sequence of the Orbit protein. Consensus sites for phosphorylation by p34cdc2 are shaded. Conserved sequence motifs shared with two related proteins from human, KIAA0622 and KIAA0627 (Ishikawa *et al.*, 1998) and two related proteins from *C. elegans*, R107.6 and ZC84.3 (Wilson *et al.*, 1994) are underlined. Two possible GTP-binding motifs, GGGTGTG identical to the GTP binding site in the FtsZ protein in *E. coli* and NKLD which matches a consensus motif for a GTP binding site in the GTPase family, are indicated by double underlining. A basic domain with a predicted pI of 11.0 and flanking short acidic regions with pI of 4.1 and 4.3, respectively are shown in boxes.

Figure 8. HR1 and HR2 domains of Orbit protein, human KIAA0622 and *C. elegans* R107.6.

(A) Schematic representation of ORBIT (1492 amino acids), KIAA0622 from human (1289 amino acids) (Ishikawa *et al.*, 1998; Genebank BAA31597) and R107.6 from *C. elegans* (1080 amino acids; Genebank CAA78472). A region from residue 290 to residue 1068 in Orbit shows 32% identity (48% similarity) of amino acid sequence to a corresponding region of KIAA622 and shows 23% identity (46% similarity) to a corresponding region of R107.6. We designated this evolutionarily conserved region of Orbit protein as HR1. Another region from residue 1291 to residue 1471 within Orbit was

designated as HR2 because of its 34% identity (59% similarity) and 21% identity (43% similarity) to corresponding regions of KIAA0622 and R107.6, respectively. Both a basic domain and two serine-rich domains characteristics of HR1 in Orbit are also conserved in corresponding positions of each related protein.

(B) Alignment of four conserved sequence motifs within the HR1 domains and one sequence motif in the HR2 domain. Five conserved sequence motifs in Orbit shared with the related proteins; KIAA0622 and KIAA0627 (Ishikawa *et al.*, 1998; Genebank BAA31602) from human, R107.6 and ZC84.3 (Wilson *et al.*, 1994; Genebank QQ3609) are listed. One conserved sequence motif from residue 326 to residue 350 shows considerable similarity to the peptide sequence of human MAP4 (Genebank NP 002366) that is involved in microtubule-binding (Olson, 1995). Another conserved sequence motif from residue 479 to residue 506 indicates a similarity to a sequence in a microtubule associated protein, STU1 from budding yeast (Pasqualone and Huffaker, 1994; Genebank P38198). Identical residues are in solid boxes and conservative amino acid changes are in light shaded boxes.

Figure 9.

(A) Levels of Orbit protein are greatly reduced in *orbit* mutants.

Equivalent amounts of total protein extract corresponding to three wild-type ovaries (lane 1); ten $orbit^{1}$ /$orbit^{1}$ ovaries (lane 2); ten wild-type larval brains (lane 3); and twenty $orbit^{3}$/*Df(3L)*$orbit^{2}$ larval brains (lane 4) were blotted, detected with an affinity-purified Orbit antibody. The antibody recognizes a major band of 165 kDa that is reduced in amount in extracts from $orbit^{1}$ ovaries and is barely detectable in extracts of $orbit^{1}$ larval brains.

(B) Orbit co-purifies with microtubules.

Western blot of the fractions obtained during the purification of microtubules from wild-type embryos (See Materials and Methods). The blot was probed with anti-Orbit (diluted 1:1500) and anti-tubulin (diluted 1:4) antibodies. (lane 1) 20 μg of crude embryonic protein extract, (lane 2) 20 μg of protein from the supernatant fraction after the centrifugation through sucrose, (lane 3) 20 μg of proteins removed from the microtubule pellet by a 250 mM NaCl wash, (lane 4) 10 μg of the final microtubule fraction. (C & D) Orbit binds to microtubules in the presence of GTP but not its non-hydrolysable analogue GTP-γ-S.

Recombinant Asp (lanes 1), Orbit protein (lanes 2), and bovine serum albumin (BSA) (lanes 3) were transferred to PVDF membranes, incubated with the indicated nucleotides, and subsequently polymerised microtubules. Binding of microtubules was assessed using anti-β-tubulin (Materials and Methods). (E-G), Orbit binds to microtubules in solution in the presence of GTP (E), but not GDP (F) or GTP-γ-S (G). Soluble Orbit protein was incubated with different concentrations of microtubules (as indicated on the figure) in the presence of GTP (E), GDP (F) or GTP-γ-S (G). Polymerized microtubules were recovered by centrifugation. Presence of Orbit in the microtubule pellet and the supernatant was assessed by immunoblot using anti-Orbit antibody.

Figure 10. Immunolocalisation of Orbit protein during the nuclear division cycle in syncytial blastoderm embryos. Wild-type syncytial embryos were fixed and stained by indirect immunofluoresence to visualize chromosomes, microtubules and Orbit proteins. Simultaneous staining for DNA with propidium iodide and microtubules with an anti-tubulin antibody, YL1/2 and rhodamine-conjugated goat anti-rat IgG are shown in red in the left column and merged images in the right column. Staining of Orbit using the primary antibody and FITC-conjugated goat anti-rabbit IgG is shown in the middle column and in the merged images. The mitotic phases are (A) prophase, (B) metaphase, (C) anaphase, (D) telophase and an interphase figure is presented in (E). Bar, 10 mm.

EXAMPLES

Materials and Methods

Drosophila *stocks and genetic analysis*

Maintenance of stocks and mating experiments were performed on standard yeast-cornmeal-agar medium. All genetic analyses were carried out at 25°C.

Immunofluorescent staining of embryos

For simultaneous visualization of microtubules, centrosomes and chromosomes, embryos from wild-type or mutant flies were fixed and stained as described by Gonzalez and Glover (1993). Microtubules were detected with the rat anti-tubulin antibody YL1/2 (Sera-Lab)

and FITC-conjugated anti-rat IgG antibody (Jackson Laboratories) as a primary and secondary antibody, respectively. Centrosomes were simultaneously revealed with the polyclonal rabbit serum, Rb188 (Whitfield *et al.*, 1988) and with Cy5-conjugated anti-rabbit IgG antibody (Jackson Laboratories). DNA was stained with 1 μg/ml of propidium iodide. For simultaneous immunolocalization of tubulin and Orbit, dechorionated embryos were fixed in freshly prepared 4 % paraformaldehyde in buffer B (45 mM KCl, 15 mM NaCl, 10 mM phosphate buffer at pH 6.8) at room temperature for 5 min and fixation continued for 25 min at 4°C. We used an affinity-purified rabbit antibody against Orbit 1-654 residues (See below) diluted 1:100 in PBS with 0.1 % Triton X-100 and the rat YL1/2 antibody diluted 1:20 as primary antibodies. Staining was revealed using FITC-conjugated anti-rabbit IgG antibody and Texas red-conjugated anti-rat IgG antibody. DNA was stained with 1 μg/ml of propidium iodide. The preparations were observed using either an MRC600 or MRC1024 laser confocal microscope (Bio-Rad). Images were processed and merged in pseudocolour using Photoshop version 5 (Adobe Systems).

Cytological analysis of larval CNS and chromosome in situ *hybridisation*

As every *orbit* chromosomes were balanced by *TM6C ,Tb Sb,* we selected the homozygotes for *orbit* mutations as non-*Tb* larvae. Wild-type and mutant larval CNSs were fixed and squashed in aceto-orcein according to the method previously described (Inoue and Glover, 1998). Immunostaining of whole mount preparations of larval CNS was carried out as described by Gonzalez (1993). For *in situ* hybridisation, polytene chromosomes were prepared from salivary glands of late third instar larvae heterozygous for *orbit*^{1} and wild-type. Biotin-labelled probe was prepared from *P-lacW* plasmid. Hybridisation procedures and signal detection were performed as described by Deak *et al.* (1997). The specimens were examined using phase contrast optics and hybridisation signals were assigned to chromosome bands referring the salivary chromosome maps described (Heino *et al.*, 1994).

Reversion analysis of orbit^{1}

Reversion analyses to test whether the *P-lacW* integrated at 78C is responsible for sterility and mitotic phenotypes of *orbit*^{1} were carried out by mating *y w; orbit*^{1}*/TM3, Sb Δ2,3* dysgenic males with *w; sr e Pr ca /TM6B, Hu Tb* females. A total of 27 *w*$^{-}$ progenies were

scored among 147 F1 flies with e^+ *Hu* or e^+ *Pr* markers. Each w^- fly was individually backcrossed with *y w*; *orbit*^{1}/*TM6C* flies. From 27 independent crosses, females trans-heterozygous for each potential revertant allele without the w^+ marker over *orbit*^{1} were selected and tested for ability to produce viable progeny. Of the 27 w^- derivatives of *orbit*^{1}, 22 turned out to be phenotypic revertants. To confirm the correlation of the phenotypic reversion of *orbit*^{1} with the loss of the *P-lacW*, we established homozygous lines of three phenotypic revertants, *orbit*^{2}, *orbit*^{3}, and *orbit*^{4}, and then examined genomic DNA organization by Southern hybridization using rescued fragments flanking both sides of the *P-lacW* element as probes. It was confirmed that all three revertants are imprecise and have internal deletions in the *P-lacW* sequence.

Northern analysis of orbit *mRNAs*

Total RNA was extracted from wild-type animals at various stages or *orbit*^{1} homozygous flies as described in Chomczynski, 1987 using Trizol (Life Technologies) instead of acidic guanidinium thiocyanate-phenol. Frozen samples were ground into a powder in liquid nitrogen to extract RNA, using a pestle and mortar. 10 volumes of Trizol and one fifth volume of chloroform were mixed with the powder. RNA was precipitated by adding iso-propanol. 20 µg of total RNA was separated by electrophoresis in a 1.0 % agarose gel containing 2.2 M formaldehyde and then transfered to Hybond N^+ membrane (Amersham) by capillary blotting in 10 X SSC. Hybridisation was performed at 42°C in 50% formamide, 1% SDS, 1 M NaCl, 100 µg/ml heat denatured salmon sperm DNA. The size of transcripts was estimated by a comparison with RNA molecular markers (Life Technologies). Membranes were rehybridised with a *Drosophila rp49* cDNA (O'Connell and Rosbash, 1984) as a control for loading.

P element mediated rescue

Genomic DNA fragments of 1.3 and 1.4 kb flanking the *orbit*^{1} insertion were isolated by plasmid rescue as previously described (Deak *et al.*, 1997). These fragments were used as probes to isolate several cosmid clones from the European *Drosophila* Genome Project cosmid library. The 14 kb *Bam*HI fragment of cosmid 193F11 containing the *orbit* transcription unit was used for germ line transformation experiments.

P element mediated germline transformation and rescue experiments.

A 14 kb *Bam*HI fragment of cosmid 193F11 containing the *orbit* transcription unit was subcloned into a transformation vector pUAST (Brand, 1994) by replacing a short *Bam*HI fragment within the vector. The resulting plasmid was microinjected at 18°C into embryos derived from *w; Δ2,3* females crossed with *w ; sr e Pr /TM6* to generate transgenic flies. Three w^+ transformats were recovered from 97 fertile G_0 adults and it was confirmed that they contain the intact transgene by genomic Southern hybridization. All transformed lines have the transgene on the third chromosome necessitating the generation of a recombined third chromosome carrying one transgenes (mapped to 3-56.5) and an $orbit^1$ mutation for rescue experiments. Females of genotype *w*; *ru h th st* $orbit^1$ / P[w^+ $orbit^+$] *sr e Pr* were crossed to *ru h th st cu sr e ca /TM3* males. Among the F1 progenies, seven males with recombined chromosomes carrying *th* and *sr* were selected and mated to *w*; $orbit^3$/*TM6C* females. Offspring containing each recombined chromosome and $orbit^3$ chromosome were examined by Southern hybridization for the presence of *Bam*HI RFLPs characteristic of of $orbit^1$, $orbit^+$ on the transgene and $orbit^3$. One of selected seven recombined chromosomes turned out to be *ru h th* $orbit^1$ P[w^- $orbit^-$] chromosome. Both a reduced viability and a sterility in $orbit^1/orbit^3$ females and males were fully rescued in *ru h th* $orbit^1$ P[w^+ $orbit^+$]/$orbit^3$ flies. An analogous procedure was followed to examine an ability of the transgene to rescue a lethality of $orbit^3/orbit^2$. Females of genotype *w*; $orbit^3$ /P[w^+ $orbit^+$] *sr e Pr* were crossed to *w*; *ru h th st cu sr e ca /TM3* males. Flies carrying the transgene were first selected by w^+ marker in the next generation. Then, after individual lines were established, flies from individual lines were examined by Southern hybridization on the basis of a RFLP of the 14kb *Bam*HI fragments containing *orbit* locus between $orbit^+$ and $orbit^3$ to identify lines with a recombined chromosome carrying $orbit^3$ and P[w^+ $orbit^+$]. Females of genotype *w*; $orbit^3$ P[w^+ $orbit^-$] *sr e Pr/TM6C* were mated to *w*; $orbit^2$/*TM6C* males. Among the F1 progenies, expected numbers of Sb^+ *Pr* flies were scored and those flies showed normal fertility at both sexes.

Preparation of anti-Orbit antibodies and Western blot analysis

A 2.0 kb *Eco*RI fragment of the cDNA clone pOrb1 was inserted in frame into the *Eco*RI site of the expression vector pGEX-2T (Pharmacia). The resulting plasmid expresses a fusion protein of a polypeptide corresponding to amino acids 1 - 632 and a stretch of 10 amino acids from the 5' untranslated region of the cDNA with the carboxyl terminus of glutachione S-transferase (GST) protein. The plasmid was introduced into *E. coli* XL-1 cells. The soluble GST-Orbit fusion protein was purified on a glutathione-Sepharose (Pharmacia) column. Antiserum was prepared by injecting rabbits as described (Harlow and Lane, 1988). We affinity-purified antibodies specific to the Orbit protein from antiserum with GST-Orbit conjugated Sepharose after preabsorption with GST conjugated Sepharose. For Western blot analysis, Canton-S females or *orbit*^{1} females were dissected and approximately equal volumes of ovaries were collected. Generally 10 larval wild-type brains or an approximately equal volume of brains from *orbit*^{3} / *Df(3L)orbit*^{2} third instar larvae were collected separately. Samples containing approximately 20 µg of protein were electrophoresed and were transferred to a PVDF membrane (Bio-Rad). To detect Orbit, the blots were incubated with the affinity-purified antibody diluted 1:1500, followed by incubation with horeseradish peroxidase-conjugated anti rabbit IgG.

Microtubule purification and binding assays

Microtubules were purified from 0-3h-old *Drosophila* embryos essentially as described previously (Saunders, *et al.*, 1997). About 3 ml of embryos were homogenized with a Dounce homogenizer in 2 volumes of ice-cold lysis buffer (0.1M Pipes/NaOH, pH6.6, 5mM EGTA, 1mM $MgSO_4$, 0.9M glycerol, 1mM DTT, 1mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin). The microtubules were depolymerized by incubation on ice for 15 min, and the extract was then centrifuged at 16,000 g for 30 min at 4°C. The supernatant was recentrifuged at 135,000 g for 90 min at 4°C. Microtubules in this later supernatant were polymerized by addition of GTP to 1mM and taxol to 20 µM and incubation at room temperature for 30 min. A 3 ml aliquot of the extract was layered on top of 3 ml 15% sucrose cushion prepared in lysis buffer. After centrifuging at 54,000g for 30 min at 20°C using a swing out rotor, the microtubule pellet was resuspended in lysis buffer.

Microtubule overlay assays were performed as previously described (Saunders *et al.*, 1997). 500 ng per lane of recombinant Asp (pAsp36), recombinant Orbit (see above), and bovine serum albumin (BSA, Sigma) were fractionated by 10% SDS-PAGE and blotted onto PVDF membranes (Millipore). The membranes were preincubated in TBST (50mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% low fat powdered milk (LFPM) for 1 h and then washed 3 times for 15 min in lysis buffer. The filters were then incubated for 30 minutes in lysis buffer containing either 1 mM GDP, 1 mM GTP, or 1 mM GTP-γ-S. MAP-free bovine brain tubulin (Molecular Probes) was polymerised at a concentration of 2 μg/ml in lysis buffer by addition of GTP to a final concentration of 1 mM and incubated at 37°C for 30 min. The nucleotide solutions were removed and the buffer containing polymerised microtubules added to the membanes for incubation for 1h at 37°C with addition of taxol at a final concentration of 10 μM for the final 30 min. The blots were then washed 3 times with TBST and the bound tubulin detected using standard Western blot procedures using anti-β-tubulin antibodies (Boehringer Manheim) at 2.5 μg/ml and the Super Signal detection system (Pierce).

Microtubule Binding Assays

Microtubules were polymerized with taxol in the absence of GTP as described in the previous section. Different concentrations of tubulin were used. Bacterially expressed Orbit was diluted to ng/μl in microtubule lysis buffer (see previous section) and centrifuged for 30 min in a refrigerated Eppendorf centrifuge at top speed in order to remove any insoluble protein. This was mixed with the microtubule preparation (final volume of 20 μl) and incubated for 30 min at 37°C in the presence of GTP, GDP, or GTP-γ-S (1 mM each). Microtubules were sedimented by centrifugation for 30 min in an Eppendorf centrifuge and both the pellet and the supernatant were saved. The pellet was washed twice with 200 μl of lysis buffer and loaded, together with the supernatant on 10% polyacrylamide by staining the membranes with Ponceau S (Sigma Chemical Co.). Presence of Orbit in the microtubule pellet and the supernatant was assessed by immunoblot using anti-Orbit antibody.

Results

Example 1 - Identification of a novel locus required for a proper progression of nuclear division at syncytial stage in *Drosophila*.

To isolate novel mutations which influence nuclear divisions in syncytial blastoderm embryos, we have screened a large collection of recessive semi-lethal or lethal mutations isolated following mobilization of a *P-lacW* element on the third chromosome (Deak *et al.*, 1997). First, we selected female sterile or very poorly female fertile lines from the collection. Then, early stage embryos derived from homozygous females crossed to heterozygous males in each stock were collected and stained with Hoechst 33258. The nuclear size, morphology and distribution was examined carefully by fluorescence microscopy. We identified the initial mutation, *orbit*^{1} as a maternal-effect mutation which resulted in an uneven nuclear density within the syncytial blastoderm embryos derived from the homozygous females.

One of the features of *orbit*^{1}-derived embryos is that the homozygous females laid less than 10% of the eggs produced by the wild type females, although the external morphology of the eggs was indistinguishable from that of wild type eggs. More than 90% of the syncytial stage embryos from the homozygous females showed a perturbation in the uniform distribution of the nuclei. 10 to 20% of the embryos from the homozygous females crossed to the heterozygous males underwent cellularisation, at least partially but only less than 1 % of total embryos hatched. Nuclei of a larger size and which were stained more brightly with Hoechst than surrounding nuclei were frequently observed in the mutant syncytial stage embryos suggesting that they contained more than a diploid amount of DNA (Figure 1C, broad arrowheads). Frequently pairs of sister nuclei were formed which appeared to result from incomplete separation or nuclear fusion (Figure 1C, narrow arrowheads). Interphase nuclei joined by chromatin bridges were also occasionally apparent in the syncytial embryos. Figure 1D illustrates a typical field from a mutant embryo at cycle 10, in which two mitotic figures indicated by the arrowheads have failed to undergo bi-polar segregation of the chromatids. By contrast most of the surrounding nuclei were at a late anaphase stage. These abnormal interphase and mitotic figures suggests that failure of proper chromosome segregation and fusion of two daughter nuclei at telophase occurred in syncytial embryo-derived *orbit*^{1} females. It is likely that such

defects may result in the frequent production of polyploid nuclei the *orbit*^{1}-derived syncytial embryos.

The existence of these abnormal mitotic figures prompted us to examine the distribution of spindle microtubules and centrosomes in the mutant syncytial embryos by immunostaining. In the field shown in Figure 2C, there are regions devoid of nuclei, in which free centrosomes that have dissociated from nuclei have formed asters of microtubules. Tripolar mitotic spindles would also be observed in the mutant embryos. Figure 2D shows a typical anaphase field in *orbit*^{1}-derived embryos, in which there were curved microtubules appeared organised by incompletely separated centrosomes. It is likely that such curved spindle microtubules persist in the disoriented midbodies at telophase (Figure 2F). Moreover, it is noted that bundles of microtubules sometimes appeared to be wavy in shape (Figure 2E), even though the spindle microtubules were organized by well-separated centrosomes. The chromatids in some of the mitotic figures associated with wavy spindles were not completely separated but appeared to be left at the midzone between the two poles. It can be speculated that reduction of the maternal gene activity by the hypomorphic mutation *orbit*^{1} resulted in a loss of spindle integrity and incomplete separation of centrosomes in syncytial blastoderm embryos, and that these spindle defects may cause failure of chromosome segregation.

orbit^{1} females had a pair of degenerated small ovaries containing fewer numbers of egg chambers, and indeed laid fewer eggs, than the wild-type females. As the homozygous females aged, fewer embryos were produced. One third of the total embryos derived from the *orbit*^{1} females possessed no nuclei. These phenotypes suggest a defect during premeiotic divisions and/or in female meiotic processes. Homozygous males for *orbit*^{1} were also sterile. The homozygotes were fully viable except that development was delayed by a couple of days under crowded culture conditions. No other adult abnormal morphologies to suggest cell cycle defects were associated with *orbit*^{1} homozygotes.

The sterility of the homozygotes in both sexes can be restored after *P-lacW* excision. *In situ* hybridization to polytene chromosomes using the P element sequence as a probe, localized the integrated P element to a single cytological site, 78C. Recombination

mapping using eight visible markers on the third chromosome mapped the *orbit* locus between st and cu to 46.6, which is a good correlation with the *P-lacW* insertion site. In addition deficiency mapping represented in Figure 5A placed the *orbit* locus in the cytological interval 78B3 to 78C2, which is proximal to *Df(3L)Pc12h* breakpoint and distal to *Df(3L)Pc* breakpoint. Therefore, it can be concluded that the *P-lacW* insertion at 78C is responsible for the *orbit*^{1} phenotypes.

Hemizygotes for *orbit*^{1} exhibited abnormal adult external morphologies typical of several cell cycle mutants such as slightly roughened eye, thin and short bristles in addition to reduced viability at the adult stage. Also, notched wings were less frequently scored. This indicates a hypomorphic nature for *orbit*^{1} and a requirement of *orbit* gene activity for later imaginal disc development.

Example 2 - Mutations at the *orbit* locus lead to an accumulation of polyploid cells with hyper-condensed chromosomes in the larval CNS.

To investigate the effects of *orbit* mutations on the progression of mitosis in dividing somatic cells, squashed preparations of the larval CNS were stained with aceto-orcein. The most striking feature of mitotic figures in the larval CNS cells their hyperploidy cells. 6% of total metaphase cells in the larval CNS from *orbit*^{1} homozygotes contained more than a diploid complement of chromosomes (Table 1). Hyperploid cells up to octoploid were observed in the mutant CNS. Hyperploid cells can be generated either by a failure of chromosome segregation or cytokinesis. In the case of *orbit*^{1}, most of the polyploid figures contained hyper-condensed chromosomes (Figure 3G, Table 1). 18% of diploid cells also contained hyper-condensed chromosomes. The mitotic index was also almost three times higher, in the orbit larval CNS, than in wild type (Table 1). Consistent with the higher mitotic index, the proportion of metaphase to anaphase in the diploid cells was also two times higher than that of wild type.

These results indicate that dividing cells in the mutant larval CNS were arrested or delayed at metaphase. These mitotic phenotypes suggest that the production of hyperploid cells might result from a failure of chromosome segregation rather than cytokinesis. This is

consistent with the abnormal morphologies of the spindle microtubules seen in the *orbit* syncytial blastoderm embryos. These cytological phenotypes are closely similar to those in mutants for the bipolar kinesin, KLP61F required for centrosome separation at prophase and which contributes to pole-pole separation and spindle elongation at late stage of mitosis.

In addition to polyploid figures with hypercondensed chromosomes, we observed less frequently abnormal mitotic figures, examples of which are presented in Figure 3D. These correspond to the circular mitotic figures reported in *mgr* or *aurora* mutants (Gonzalez, 1988, Glover *et al.*, 1995). We scored mitotic figures as circular only when all the centromeres of the major chromosomes were aligned around the circle with all arms oriented toward the periphery and the fourth chromosomes located at the centre. We have not scored any mitotic figures which agreed strictly with the above definition of the circular mitotic figures among observations of more than 500 mitotic cells in wild type larval CNS. Even though the proportion of the circular mitotic figures was not remarkably high, it is significant. Monopolar anaphase-like figures like that shown in Figure 3E were also observed in *orbit*^{1} larval CNS cells.

In hemizygotes for *orbit*^{1} over *Df(3L)Pc-9a*, the proportion of polyploid cells increased by up to 13 %. In addition, both the proportion of diploid metaphase figures containing hyper-condensed chromosomes and the ratio of metaphase to anaphase also increased (Table 1). Thus, cytological examination of *orbit*^{1} confirmed the hypomorphic nature of this allele.

Table 1. The mitotic phenotypes of larval CNS cells from *orbit* mutants.

genotype	fields[a]	numbers of polyploid figures (%)[b]	numbers of hyper-condensed figures (%)[c]	numbers of CMFs (%)[d]	metaphase to anaphase ratio	mitotic index[e]
+ / +	501	0 (0)	0 (0)	0(0)	3:1	0.9
orbit^{1}/*orbit*^{1}	341	44 (6)	118(18)	21(3)	6:1	2.4
orbit^{1}/*Df(3L)Pc-9a*	488	120(13)	248(32)	38(5)	10:1	2.0
orbit^{1}/*orbit*^{2}	375	165(16)	345(40)	25(3)	11:1	3.0
orbit^{1}/*orbit*^{3}	444	128(10)	448(34)	38(3)	20:1	3.4
orbit^{1}/*orbit*^{4}	188	75(15)	150(35)	15(4)	21:1	2.8

[a]Microscope field defined by photographic viewfinder grid using X60 objective.

[b]Polyploid and aneuploid metaphase figures were counted and the percentage of those figures per total metaphase figures are presented in parenthesis.

[c]The percentage of diploid metaphase figures containing hyper-condensed chromosomes per total metaphase figures are shown in a parenthesis .

[d]The percentage of circular metaphase figures (CMF) per diploid metaphase.

[e]Average number of mitotic cells per optical field.

To examine the effects of null mutations of the *orbit* gene on mitotic progression, we have isolated three lethal mutations *orbit*^{2}, *orbit*^{3} and *orbit*^{4} by imprecise excision of the integrated *P-lacW* element in *orbit*^{1}. *orbit*^{2} and *orbit*^{4} also affect the neighbouring *asparaginase synthetase* gene (see figure legends), whereas *orbit*^{3} deletes only *orbit* sequences. Most of the homozygotes for *orbit*^{3} and *orbit*^{4} or trans-heterozygotes between these two alleles died during the larval stage at least before the mature larval stage. Hemizygotes for *orbit*^{2} over *Df(3L)Pc-9a* survive until third instar larvae to early pupae, although the homozygotes died at an early larval stage due to another spontaneous lethal mutation not included by *Df(3L)Pc-9a*. Some of the trans-heterozygotes between *orbit*^{2} and *orbit*^{3} or between *orbit*^{2} and *orbit*^{4} can survive until early pupal stage. These combinations facilitated further cytological analyses of lethal mutants. The third instar larvae of these lethal mutants lacked imaginal discs and had a small larval CNS.

Extremely high proportions of hyperploid cells was a common characteristic of these lethal mutants. 81% to 91% of total metaphase figures from the lethal mutants contained more than diploid complement of chromosomes that were hyper-condensed (Table 2). Not only the proportion of hyperploid cells but the extent of hyperploidy also increased in these lethal mutants. More than 32 % of the polyploid figures seen in the lethal mutants possessed greater than an 8N complement chromosomes. Figure 3H shows a typical mitotic figure of extremely hyperploid cells from *orbit*^{2}/*orbit*^{3} trans-heterozygotes containing more than one hundred extremely hyper-condensed chromosomes. These lethal mutants showed no anaphase figures and a lower mitotic index reflecting an extensive inhibition of chromosome segregation and cell division (Table 2). The cytological phenotypes such as the proportion of hyperploid cells in the trans-heterozygotes between each lethal allele and *orbit*^{1} are comparable to those of *orbit*^{1} hemizygotes over *Df(3L)Pc-9a*. This indicates the amorphic nature of these three lethal alleles. The proportion of the circular metaphase figures in *orbit*^{1} hemizygotes for or trans-heterozygotes betwen *orbit*^{1} and amorphic alleles did not increase significantly to compared to that in *orbit*^{1} homozygotes. We were not able to find diploid circular metaphase figures among a total of 300 mitotic figures from lethal mutants. This may reflect the unusually low proportion of diploid cells among the mutant CNS cells or alternatively it may suggest that some aspect of *orbit* gene activity might be required for formation of the circular metaphase figures.

Table 2. Polyploid mitotic figures observed in larval CNS cells from *orbit* lethal mutants.

genotype	fields[a]	numbers of polyploid cells(%)	numbers of >8N polyploid (%)[b]	percentage of hyper-condensed diploid cells (%)[c]	percentage of anaphase figures (%)	mitotic index[d]
orbit^{2}/*Df(3L)Pc-9a*	324	59(85)	29(50)	100	0	0.21
orbit^{2}/*orbit*^{3}	467	87(81)	46(43)	100	0	0.23
orbit^{2}/*orbit*^{4}	555	79(91)	47(46)	100	0	0.16
orbit^{3}/*df(3L)Pc-9a*	98	130(84)	42(32)	100	0	1.6

[a]Microscope field defined by photographic viewfinder grid using X60 objective.

[b]The percentage of polyploid figures containing more than 8N chromosome complements per total polyploid figures is presented in a parenthesis.

[c]The percentage of diploid metaphase figures containing hyper-condensed chromosomes is in a parenthesis.

[d]Average numbers of mitotic cells per optical field.

Example 3 - Monopolar mitotic spindles in a hemispindle structure.

As described above, since both observation of the syncytial blastoderm embryos derived from *orbit*^{1} females and cytological examination of *orbit*^{1} larval CNS cells suggested spindle abnormalities. Mitotic spindles and centrosomes in the larval neuroblasts were observed directly by immunostaining of whole mount preparations of the *orbit*^{1} larval CNS with anti-tubulin antibody and with an antibody against the centrosome-associated antigen CP190. As shown in Figure 4A, bipolar spindles organized by two fully separated centrosomes are observed at metaphase in the wild-type larval CNS cells. In contrast to wild-type, about 10% of mitotic figures observed in the larval CNSs from the hypomorphic mutant *orbit*^{1} were polyploid. Either multipolar spindles (Figure 4B) or bipolar spindles were associated with polyploid chromosomes. Interestingly, unlike monopolar mitotic figures seen in whole mount larval brains obtained from *asp* or *klp61F* mutants, the chromatids appeared to be pulled towards a single pole (Figure 4C). Other circular mitotic figures in which a single centrosome was located at the centre of the circle of chromosomes were also observed at a lower frequency (Figure 4E).

These figures may be similar to the circular figures seen in the *aurora* mutant (Glover *et al.*, 1995). It should be noted that the centrosome lies on almost the same confocal plane as the chromosomes. These figures correspond to the circular mitotic figures seen in squashed preparations of larval CNS (Figure 3D). It is possible to speculate that the multipolar spindles observed in the polyploid cells could be organized by multiple centrosomes which may result from either a failure of chromosome segregation or cytokinesis.

Example 4 - Molecular cloning of *orbit*

Reversion analysis of *orbit*^{1} confirmed that the *P-lacW* element at 78C is responsible for the mutant phenotype. The flanking genomic DNA adjacent to the *P-lacW* insertion was cloned by plasmid rescue. A restriction map of the 20 kb genomic region surrounding the insertion is shown in Figure 5D. Northern blot analysis using the 6 kb HindIII genomic fragment spanning the insertion as a probe revealed two transcription units around the P element insertion site. One transcription unit which lies toward the right side of the insertion encodes a transcript of 6.5 kb. Another transcription unit on the left side of the insertion encodes a *Drosophila* homologue of asparagine synthetase. A combination of sequence analysis of the cDNAs of both transcription units and the genomic DNA fragments around the insertion revealed that the P element responsible for the *orbit* phenotypes was inserted 503 bp upstream of the first ATG of the transcription unit encoding the 6.5 kb transcript. In adult males, a 6.0 kb transcript was also expressed from this gene.

Neither transcript could be detected in adult females or males homozygous for *orbit*^{1} by Northern blot hybridization (Figure 6B). Moreover, Southern blot analysis of genomic DNA from the heterozygotes for the null allele *orbit*^{3} over a balancer chromosome showed that this allele was associated with a 3 kb long deletion within the gene for the 6.5 kb transcript extending from the 5' regulatory region into the coding region (Figure 5E) but not extending to flanking genes. In the two allele *orbit*^{2}, a deletion extended from the 5' UTR of the gene for the 6.5 kb transcript into the flanking *asparagine synthetase* gene, and in *orbit*^{4} the deletion extended from the coding region of the 6.5 kb gene into the *asparagine synthetase* gene.

To confirm that the gene encoding the 6.5 kb transcript is indeed responsible for the mitotic phenotypes of the *orbit*, we carried out a germline transformation experiment. We introduced the 14 kb *Bam*HI fragment of genomic DNA from wild-type flies containing the entire 6.5 kb transcription unit and the 3' third of the *asparagine synthetase* gene into the transformation vector (See Materials and Methods). Transformants carrying this transgene fully rescued the female sterility and viability in *orbit*^{1}/*orbit*^{3} transheterozygotes allowing us to conclude that this gene for the 6.5 kb transcript corresponds to the *orbit* gene. The 6.5 kb *orbit* transcript throughout *Drosophila* development and in adult flies

(Figure 6A). In adult males, a 6.0 kb transcript was also found in addition to the common 6.5 kb transcript.

Example 5 - The *orbit* gene encodes a novel conserved protein of 170 kDa with a highly basic domain.

We determined a total of 5959 bp of orbit cDNA sequences by combining the DNA sequences of several overlapping cDNA clones. The first ATG consistent with a *Drosophila* translation initiation consensus is found at position 769 and a poly(A) additional signal AATAAA lies at position 5892. This cDNA encodes a predicted polypeptide consisting of 1492 amino acid residues with a molecular weight calculated as 165,420 Da. Indeed, we detected a polypeptide of almost identical molecular weight with an antibody against a GST-Orbit fusion protein in Western blot analysis using *Drosophila* extracts (See below).

This putative polypeptide does not show significant sequence homology to any known cell cycle-related proteins. The novel protein contains a highly basic region (pI=11.0) of 411 amino acids in the central region. The basic domain is surrounded by short stretches of acidic residues at both sides. Two consensus sites for phosphorylation by $p34^{cdc2}$ are present in the basic domain (Figure 7). There are two putative GTP-binding motifs in the basic domain: GGGTGTG at residue 544 is identical to the glycine-rich peptide which interacts with the guanine or phosphate groups of the bound GTP in FtsZ protein in *Escherichia coli* and closely resembles the GTP binding motif in β-tubulin. NKLD at residue 400 corresponds to the NKXD (X for any amino acid residues) consensus motif which can interact with the purine base of the bound nucleotide in the GTPase superfamily (Burns and Farrell, 1996). A comparison of the Orbit protein sequence to protein sequences in the NCBI database using the BLASTP program suggested the presence of closely related proteins from other organisms: KIAA0622 and KIAA0627 from human, R107.6 and ZC84.3 from *C. elegans* (Figure 8A). The human proteins were identified from the human genome sequencing project to determine the entire sequencing of 100 new cDNA clones encoding putative large open reading frames (Ishikawa *et al.*, 1998). Two

putative proteins, R107.6 and ZC84.3 were predicted from the *C. elegans* genome sequencing project (Wilson *et al.*, 1994).

A homologous protein in plants was also identified by the database search. A hypothetical 199 amino acid protein (AAD21767.1) predicted from genomic sequencing of *Arabidopsis thaliana* exhibited 29 % identity over 155 amino acids to the amino terminal region of the Orbit protein. Furthermore, a search of the database of the *Schizosaccharomyces pombe* genome sequencing project revealed that a significantly related protein (O42874) was also found in fission yeast as well as in metazoans. Within the hypothetical 164.1 kDa protein in fission yeast, there are three regions, residue 1 to residue 192, residue 238 to 498, and residue 505 to 589, which are 23 % , 20 %, 28 % identical to Orbit, respectively.

A comparison of the Orbit protein with the homologous proteins from human and nematode revealed two homologous regions, HR1 lying between residue 290 and residue 1068 and HR2 between residue 1093 and residue 1271, shared between these proteins. Moreover, the basic domain contained in HR1 is also a common feature of the human, nematode and fly proteins. The consensus sequences for cdc2 phosphorylation are found within or in the vicinity of basic region in these related proteins with the exception of ZC84.3. The NKXD motifs for GTP-binding are also conserved in both related human proteins. Five particularly conserved sequence motifs shared between the related proteins are shown in Figure 8B. Every conserved sequence in the HR1 region lies within the basic domain suggesting an important role for this domain. Interestingly, the homology search by the BLASTP program suggested that one of the conserved sequences (from residue 326) shows considerable similarity to the sequence involved in the binding of human MAP4 protein to microtubules (Olson *et al.*, 1995).

Furthermore, another conserved sequence (from residue 479) shows similarity to a sequence in STU1, a microtubule-associated protein of budding yeast (Pasqualone and Huffaker, 1994). It has been shown that known microtubule-associated-proteins such as MAP4 interact with microtubules via basic domains. The presence of peptide sequences with similarity to microtubule-associated proteins in the basic domain suggests a functional role for this region of the Orbit protein in binding microtubules.

Example 6 - The orbit protein associates with microtubules in a GTP dependent manner

The presence of motifs within a basic domain that have similarity to microtubule-associated protein domains suggested that the Orbit protein might itself bind microtubules and that this could explain its requirement in mitosis. To investigate potential interactions of Orbit with microtubules, we first raised a polyclonal antibody against a fusion protein between GST and a polypeptide corresponding to amino-acid residues 1- 632. The affinity-purified antibody recognizes a polypeptide of 160-170 kDa in immunoblots of extracts from ovaries or third instar larval brains (Figure 6C and 9A) that is greatly reduced in ovaries of homozygous *orbit*^{1} females, and barely detectable in the third instar larval brains from the transheterozygotes between the two amorphic alleles *orbit*^{2} and *orbit*^{3}. (Note that increased amounts of tissue extract were loaded from the mutant brains and ovaries to normalize total protein loaded). We conclude therefore that this band corresponds to the Orbit protein. The molecular weight of Orbit estimated from its electrophoretic mobility is in good agreement with the molecular weight of 165,420 Da calculated from the amino acid sequence. We then purified microtubules from *Drosophila* embryo extracts by taxol induced polymerization, followed by centrifugation, and salt washing of the pellet. We found that Orbit protein copurified with β-tubulin in this preparation and so by this criterion is a novel microtubule-associated protein (Figure 9B).

We assessed the direct binding of Orbit to tubulin in microtubule overlay assays using phosphocellulose purified MAP-free tubulin (Figure 9C & D). Recombinant Orbit protein containing the putative tubulin binding domain and the two GTP binding motifs was transferred to PVDF membranes (Materials and Methods) which were preincubated with GDP, GTP, or its non-hydrolysable analogue GTP-γ-S. Recombinant Asp protein was used as a positive control for microtubule binding and BSA as a negative control. The filters were then incubated with polymerised microtubules and binding detected using anti-tubulin antibodies. We found that this segment of Asp protein would bind microtubules irrespective of the preincubation treatment. In contrast, Orbit would bind microtubules

only when first incubated with GTP, but not with GDP (not shown) or GTP-γ-S (Figure 9C D). We conclude that to bind microtubules, Orbit must bind GTP.

To confirm the results obtained by the microtubule overlay assays, we have also assessed whether Orbit would bind microtubules in a GTP-dependent way when in solution. Microtubules were polymerized with taxol in the absence of GTP and then incubated with soluble Orbit protein in the presence of GTP, GTP, or GTP-γ-S (Fig. 9E, F, and G, respectively). Binding to microtubules was detected by Western blots after sedimentation of the tubulin polymers by centrifugation. In the presence of GTP, Orbit was found exclusively in the microtubule pellet, whereas the protein was in the supernatant when either GDP or GTP-γ-S were used. This was independent of the microtubule concentration. This substantiates the earlier conclusion that to bind microtubules, Orbit must bind GTP.

Example 7 – The Orbit protein is co-localized with tubulin during mitosis

To determine whether the Orbit protein is a component of the mitotic spindle, we performed immunostaining of syncytial blastoderm embryos using the affinity-purified antibody described above and compared the staining pattern with distribution of tubulin (Figure 10). As syncytial embryos enter mitosis at prophase, Orbit protein accumulates distinctly at the periphery of nuclei in the polar regions showing extensive colocalisation with tubulin as the spindle forms (Figure 10). Throughout metaphase to anaphase, Orbit colocalises with microtubules throughout the entire region of the mitotic spindle and its asters (Figure 10B, C). The microtubule association remains with the mid-body (Figure 10D), and some residual mid-body staining appears to remain in interphase (Figure 10E).

Example 8 - A mutation in the bipolar kinesin, KLP61F dominantly enhanced the mitotic phenotypes of the hypomorphic mutation, *orbit*^{1}.

Since the abnormal mitotic figures seen in the larval CNS cells from the *orbit* mutants were closely similar to the reported mitotic figures in the *klp61F* mutants and the subcellular localization of the Orbit protein overlapped with that of KLP61F, we examined the genetic interaction between mutations in these two genes. Heterozygotes between the

amorphic allele, *orbit*^{3} and the lethal allele, *klp61F*^{1} (Heck, 1993) exhibited normal fertility in both sexes in addition to having an external morphology indistinguishable from wild-type. We constructed a recombined chromosome carrying *klp61F*^{1} and the hypomorphic mutation *orbit*^{1}. Heterozygotes for the double mutant chromosome and a wild-type chromosome were normal with respect to fertility, viability and adult morphology. However, 70 % of the flies which were trans-heterozygotes between the double mutations and the *orbit*^{1} alone, namely *klp61F*^{1} *orbit*^{1} /+ *orbit*^{1} , died soon after eclosion or during eclosion due to poor locomotion, although *orbit*^{1} homozygotes are fully viable. Thus, a reduction in the gene dosage of the *klp61F* gene by *klp61F*^{1} resulted in a reduced viability of the hypomorphic mutation *orbit*^{1} at adult stage.

We then examined the proportion of hyperploid cells and the extent of polyploidy in larval CNS cells from the heterozygotes between the recombined chromosome and the *orbit*^{1} chromosome to compare to those of homozygotes for *orbit*^{1} alone. The trans-heterozygotes between the double mutations and the *orbit*^{1} alone showed a significantly higher proportion of polyploid cells (25.4%) than the proportion in the *orbit*^{1} single mutant (6.0 %), whereas we scored no polyploid cells in the larvae with the recombined chromosome carrying both *klp61F*^{1} and *orbit*^{1} when heterozygous with a wild type chromosome. The proportion of polyploid cells in the trans-heterozygotes is comparable to the proportion (31.2 %) in the *klp61F*^{1} homozygotes. Not only the frequency of polyploid cells but also the proportion of highly polyploid cells containing more than an 8N complement of chromosomes (22 %) increased as a consequence of a reduction in *klp61F* as compared with a low proportion of such highly polyploid cells (2 %) in the *orbit*^{1} single mutant. Thus, the loss of one dose of the *klp61F* gene strikingly raised the proportion of polyploid cells and the degree of hyperploidy compared to homozygotes for *orbit*^{1} alone. This dominant enhancement of the *orbit*^{1} phenotypes by the *klp61F* mutation suggest a close interaction between two proteins possibly during spindle pole separation and elongation of pole-to-pole microtubules for which the bipolar kinesin KLP61F is required.

Discussion

Spindle Defects in orbit^{1} embryos

The mutant phenotype of *orbit* is suggestive of a role for the wild-type gene in the functioning of the mitotic spindle consistent with the gene product being a novel MAP. This discovery helps overcome the difficulty in interpreting mitotic phenotypes in syncytial embryos derived from homozygous mutant females. Such difficulties arise since syncytial embryos lack certain checkpoints and so aspects of mitotic cycles can continue even though other steps are blocked. This is reflected by the finding of free centrosomes in *orbit^{1}*-derived embryos that appear to be undergoing autonomous duplication cycles, as seen in many other mitotic mutants. Maternal effect mutations leading to mitotic defects are often hypomorphic, and have some residual function that allow the homozygous mothers to survive to adulthood partly assisted by a supply of wild-type gene product from the heterozygous grandmother. *orbit^{1}* is no exception to this rule, and indeed it proved possible to make amorphic alleles that show larval lethality by remobilization of the P-element responsible for the original mutation. Nevertheless the characteristic spindle defects of two types seen in *orbit^{1}*-derived embryos reflect the specific effect of diminution of the levels of Orbit protein. The branched spindles could either be an immediate consequence of failure in centrosome duplication or separation, or they could arise by capture of a free centrosome by an otherwise bipolar spindle. In either case, these defects together with the high proportion of the spindles with wavy or bent arrays of microtubules indicate a role for the Orbit protein in regulating function of spindle microtubules. Branched spindle defects are also seen in *aurora*-derived embryos thought to be defective in aspects of centrosome separation (Glover *et al.*, 1995), and as with *orbit^{1}* are often associated with the generation of what appear to be tetraploid nuclei in the syncytial blastoderm. Such nuclei could arise either as a consequence of the failure of chromosome segregation, or a re-fusion of sister chromatids or sister nuclei after segregation. The finding of wavy and bent spindle microtubules, however, is not seen in *aurora*-derived embryos and resembles more the maternal effect phenotype described for certain alleles of *asp* (Gonzalez *et al.*, 1990). Taken together, the different aspects of the maternal effect phenotype suggest a primary defect in spindle microtubule function leading to failure of chromosome segregation.

Origins of polyploidy in the orbit *CNS*

Defective spindle microtubule function is also evident in the developing larval central nervous system of *orbit*^{1} mutants. A high frequency of cells in a metaphase like state suggests that the spindle integrity checkpoint has been activated to delay progression through mitosis. The high degree of chromosome condensation provides further evidence that the cells have been arrested at this point for some time, during which there has been continued activity of $p34^{cdc2}$. There are two characteristic features of the arrested cells in the *orbit*^{1} mutant; a low frequency of monopolar mitotic structures and also polyploid cells. The proportion of polyploid cells increases when the *orbit*^{1} mutation is hemizygous indicative of its hypomorphic nature. In the amorphic mutant combinations, monopolar figures are no longer seen and virtually all cells become polyploid and at much greater levels. Polyploid cells could arise either through a defect in chromosome segregation followed by exit from mitosis, and subsequent re-entry into the next mitotic cycle, or alternatively, there can be a failure of cytokinesis. The findings of a high mitotic index with very few anaphases, and the presence of monopolar figures strongly suggests to us that the polyploidy arises as a consequence of spindle defects leading to a failure of chromosome segregation. Of course this would not preclude some function for the Orbit protein in the late mitotic spindle, the correct structure of which is essential for cytokinesis to take place. However, the low incidence of anaphase figures in *orbit* mutants suggests that mitotic events rarely proceed to this stage.

The high levels of polyploidy attained in cells of the amorphic *orbit* mutants indicates that they have gone through repeated cell cycles without division, and precludes analysis of the primary mutant defect. The hypomorphic *orbit*^{1} mutant on the other hand, allows us to glimpse those aspects of mitosis that are most sensitive to diminished Orbit function. The observation of a low frequency of monopolar spindle structures suggests that Orbit assists in promoting the correct separation of centrosomes to form a bipolar spindle structure. However, there would seem to be other requirements for the Orbit protein in the spindle since bipolar spindles do form which then appear to undergo spindle checkpoint arrest at metaphase. Indeed the centrosome separation defect may be secondary to a spindle microtubule function. In this respect *orbit* mutants differ from *merry-go-round* (*mgr*) or *aurora* (*aur*) which appear to have a more direct role in centrosome separation. Not only is

the frequency of monopolar mitotic figures lower in *orbit* than in *mgr* or *aur*, but also monopolar structures are not seen in amorphic *orbit* mutants whereas they increase in frequency in amorphic *aur* mutants. This suggests a direct role for the Aurora protein kinase in centrosome separation such that in its absence the mitotic cycle is definitively arrested at this point. In contrast, the decrease in mitotic index and accompanying increase in levels of polyploidy in amorphic *orbit* mutants is indicative of cells continually delayed and repeatedly leaking through the spindle integrity checkpoint. The structure of the monopolar figures also differs between these mutants. In *mgr* and *aur*, the chromosomes are invariably arranged in a circle in a metaphase-like state as if under tension with their centromeres pulled towards but always at some distance from the center of the circle and the chromosome arms pulled out towards the periphery. Similar figures are seen in *orbit*^{1}, but in addition there are anaphase-like figures in which the centromeres appear to have been pulled into the immediate vicinity of a single pole. These cytological phenotypes more closely resemble those in the mutants for the kinesin-like protein, KLP61F first thought to be required for centrosome separation at prophase, but then shown by antibody injection experiments to be required for maintenance of spindle bipolarity. The orbit protein appears to be localised throughout the mitotic spindle like the KLP61F protein (Barton *et al.*, 1995), although at the em level it is apparent that KLP61F is not uniformly distributed.

Mitotic functions of microtubule-associated proteins

Orbit protein is associated with all spindle and astral microtubules at all stages of the mitotic cycle, and microtubules from embryo cytoplasm copurify with Orbit protein attached to them. The primary sequence of the Orbit protein reveals it to be a basic protein, a characteristic of microtubule-associated proteins. Moreover within these highly basic regions are motifs that strongly resemble sequences present in the vertebrate and yeast microtubule-associated proteins MAP4 and Stu1p. Aizawa and colleagues (Aizawa *et al.*, 1990) described three distinctive features in the microtubule-binding domains of MAP2, tau, and MAP4. Polypetides comprising these different domains cause microtubules assembled *in vitro* to adopt to take different shapes (Tokuraku *et al.*, 1999). Similarly, microtubules assembled in the presence of the individual neuronal MAPs, MAP1A, MAP1B, and MAP2 were also shown to adopt a variety of shapes from 'short and straight' to 'long and bendy' (Pedrotti *et al.*, 1996). Thus the bending of spindle shape seen in *orbit*^{1}

embryos may be indicative of requirement for the Orbit protein to confer a certain shape to the spindle micotubules.

Many of the first microtubule-associated proteins to be characterized were obtained from preparations of tubulin from mammalian brain, and are likely to have their primary function in the neuronal cytoskeleton. Nevertheless, it is now appreciated that some of these proteins are expressed in other tissues in which there is cell proliferation. The function of both neuronal and non-neuronal microtubules is known to be modulated by phosphorylation. The *Xenopus* homologue of MAP4, for example, is phosphorylated by both $p34^{cdc2}$ and mitogen-activated-protein kinases whereupon it looses its microtubule-binding and stabilizing properties. Expression of a mutant form of XMAP4, in which the serine residues in the phosphorylation sites are replaced by alanine residues affects chromosome movement during anaphase A suggesting that these phosphorylation events are important for the progression of mitosis. Similarly, Stathmin/Op18 is a protein that interacts with tubulin to inhibit microtubule polymerisation, and over-expression of its non-phosphorylatable forms prevents mitotic spindle assembly in tissue culture cells. It appears that mitotic chromatin can induce the phosphorylation of Stathmin/Op18 and so stimulate microtubule growth around chromosomes during spindle assembly. In this context, the conserved $p34^{cdc2}$ sites in Orbit may well play a role in regulating its function. Moreover, the abundance of serine residues within two regions of the protein may be indicative of sites for phosphorylation by other mitotic kinases such as Polo or Aurora whose consensus sites are not yet known.

Other microtubule associated protein can act through destabilising the polymers, for exmaple the Kin I kinesin, and others such as katanin can actually sever the microtubules. A significant number of microtubule-associated proteins are thought to modulate microtubule dynamics. Interphase microtubules are more stable than their mitotic counterparts which show elevated rates of turnover. This is determined by four parameters: the rate of growth and the rate of shrinkage, and the transitions from growth to shrinkage (known as catastrophes) and from shrinkage to growth (known as rescue). While the three *Xenopus* MAPs so far best characterized all localize to the spindle, they have different

effects on microtubule dynamics: XMAP215 promotes microtubule growth, XMAP230 decreases the catastrophe frequency, and XMAP310 increases the rescue frequency (Vasquez *et al.*, 1994; Andersen and Karsenti, 1997). The proportions of these proteins is similar in interphase and mitotic egg extracts, but each becomes more phosphorylated in mitosis. The total interphase MAPs promote microtubule polymerisation twice faster than the mitotic MAPs without having any effect upon the catastrophe rate (Andersen, 1998). This is taken to substantiate the hypothesis that phosphorylation of the MAPs can destabilise microtubules and make them more accessible to molecules that increase the catastrophe frequency.

The *orbit* phenotype, however, points towards the existence of microtubule-associated proteins with a specific role on the mitotic spindle. What might such a role be? In addition to modulating microtubule shape and dynamics, mitotic MAPs could assist the interactions of other proteins with the mitotic apparatus. They could for example, serve to target regulatory molecules such as protein kinases and phosphatases to specific parts of the mitotic apparatus. In fact, it has been proposed that MAP4 mediates the association of cyclin B and thereby $p34^{cdc2}$ to the mitotic spindle (Ookata *et al.*, 1995). An alternative function for MAPs would be to provide anchor points for interactions with microtubule-associated motors, in effect to provide a molecular clutch to regulate the transmission of force generated by movement upon one microtubule with respect to another.

The mutant phenotype of Orbit and the fact that the protein binds microtubules in a GTP dependent manner strongly argues for a role in the regulation of mitotic spindle dynamics. It would be of interest to determine whether there is any interaction between Orbit and the Awd protein (Abnormal wing discs) a microtubule associated NDP kinase that converts GDP to GTP. *awd* mutants display hypercondensed chromosomes typical of those seen in colchicine treated cells, suggesting activity of this enzyme is required for microtubule polymerisation. Given our present findings, however, it is also likely that the Awd protein can influence other aspects of microtubule behaviour. The availability of hypomorphic *orbit*^{1} mutants now raises the future possibility of using genetic screens to search for mutations that either enhance or suppress the *orbit* phenotype. Such mutations could

identify genes encoding proteins that interact with or regulate Orbit protein function in the mitotic spindle.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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CLAIMS

1. A polynucleotide encoding orbit or a homologue thereof.

2. A polynucleotide according to claim 1 wherein said homologue is human orbit.

3. A polynucleotide selected from:

(a) polynucleotides comprising the nucleotide sequence set out in SEQ ID No. 1 or the complement thereof.

(b) polynucleotides comprising a nucleotide sequence capable of hybridising to the nucleotide sequence set out in SEQ ID No. 1, or a fragment thereof.

(c) polynucleotides comprising a nucleotide sequence capable of hybridising to the complement of the nucleotide sequence set out in SEQ ID No. 1 or a fragment thereof.

(d) polynucleotides comprising a polynucleotide sequence which is degenerate as a result of the genetic code to the polynucleotides defined in (a), (b) or (c).

4. A polynucleotide probe which comprises a fragment of at least 15 nucleotides of a polynucleotide as defined in any one of claims 1 to 3.

5. A polypeptide which comprises the sequence set out in SEQ ID No 2, or a homologue, variant, derivative or fragment thereof.

6. A polynucleotide encoding a polypeptide according to claim 5.

7. A vector comprising a polynucleotide as defined in any one of claims 1 to 3 or 6.

8. An expression vector comprising a polynucleotide as defined in any one of claims 1 to 3 or 6, operably linked to a regulatory sequence capable of directing expression of said polynucleotide in a host cell.

9. An antibody capable of binding the polypeptide of SEQ ID. No. 2 or a fragment thereof.

10. A method for detecting the presence or absence of a polynucleotide as defined in any one of claims 1 to 3 or 6 in a biological sample which comprises:

(a) bringing the biological sample containing DNA or RNA into contact with a probe according to claim 4 under hybridising conditions; and

(b) detecting any duplex formed between the probe and nucleic acid in the sample.

11. A method for detecting a polypeptide as defined in claim 5 present in a biological sample which comprises:

(a) providing an antibody according to claim 9;

(b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said antibody is formed.

12. A polynucleotide according to any one of claims 1 to 3 or 6 for use in therapy.

13. A polypeptide according to claim 5 for use in therapy.

14. An antibody according to claim 10 for use in therapy.

15. A method of treating a tumour comprising administering to a patient in need of treatment an effective amount of a polynucleotide as defined in any one of claims 1 to 3 or 6,

16. A method of treating a tumour, comprising administering to a patient in need of treatment an effective amount of a polypeptide as defined in claim 5.

17. A method of treating a tumour, comprising administering to a patient in need of treatment an effective amount of an antibody as defined in claim 10 to a patient.

18. Use of an orbit polypeptide or homologue, derivative, variant or fragment thereof in a method of identifying a substance capable of affecting orbit function.

19. Use of an orbit polypeptide or homologue thereof, or fragment in an assay for identifying a substance capable of inhibiting mitosis.

20. A method for identifying a substance capable of binding to an orbit polypeptide or a homologue, derivative, variant or fragment thereof, which method comprises incubating the orbit polypeptide or homologue, derivative, variant or fragment thereof with a candidate substance under suitable conditions and determining whether the substance binds to the orbit polypeptide or homologue, derivative, variant or fragment thereof.

21. A substance identified by the method of claim 18, 19 or 20.

22. A substance according to claim 21 for use in a method of inhibiting mitosis.

23. A process comprising the steps of:

(a) performing the method according to claim 18, 19 or 20; and

(b) preparing a quantity of those one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

24. A process comprising the steps of:

(a) performing the method according claim 18, 19 or 20; and

(b) preparing a pharmaceutical composition comprising one or more substances identified as being capable of binding to an orbit polypeptide or homologue, derivative, variant or fragment thereof.

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(57) Abstract: Polynucleotides encoding a novel *Drosophila* gene product designated orbit and homologues thereof as well as orbit polypeptides are provided. Polynucleotide probes derived from the nucleotide sequence of orbit and antibodies that bind to orbit protein are also provided as well as assays for identifying substances that regulate orbit function.

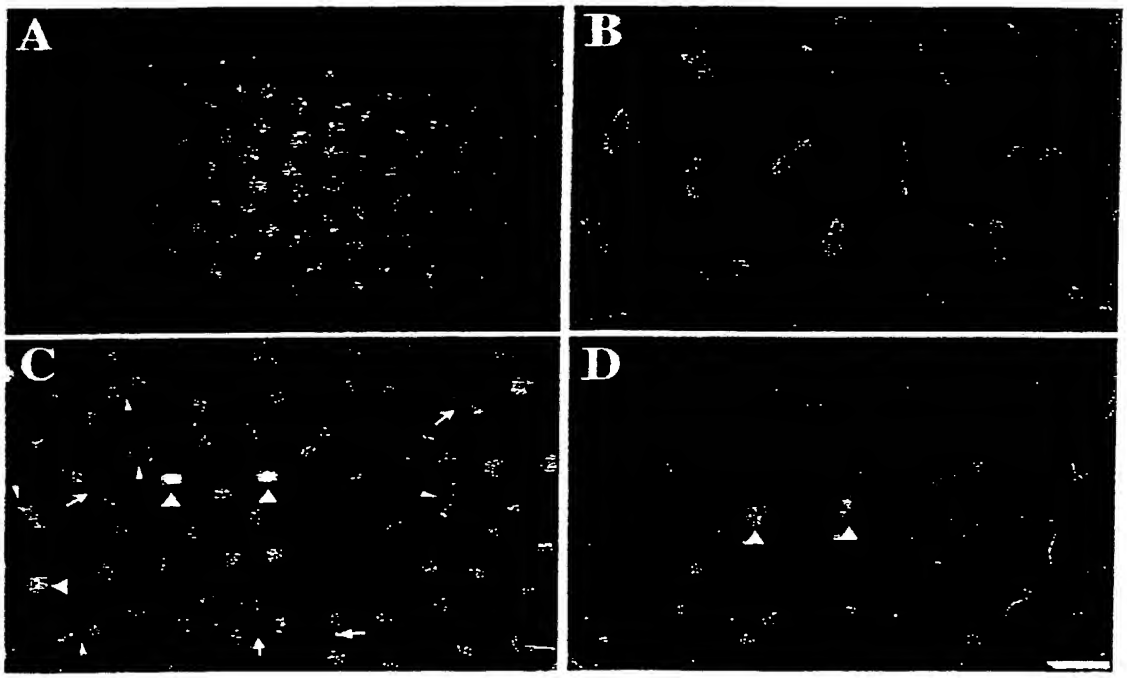


Figure 1

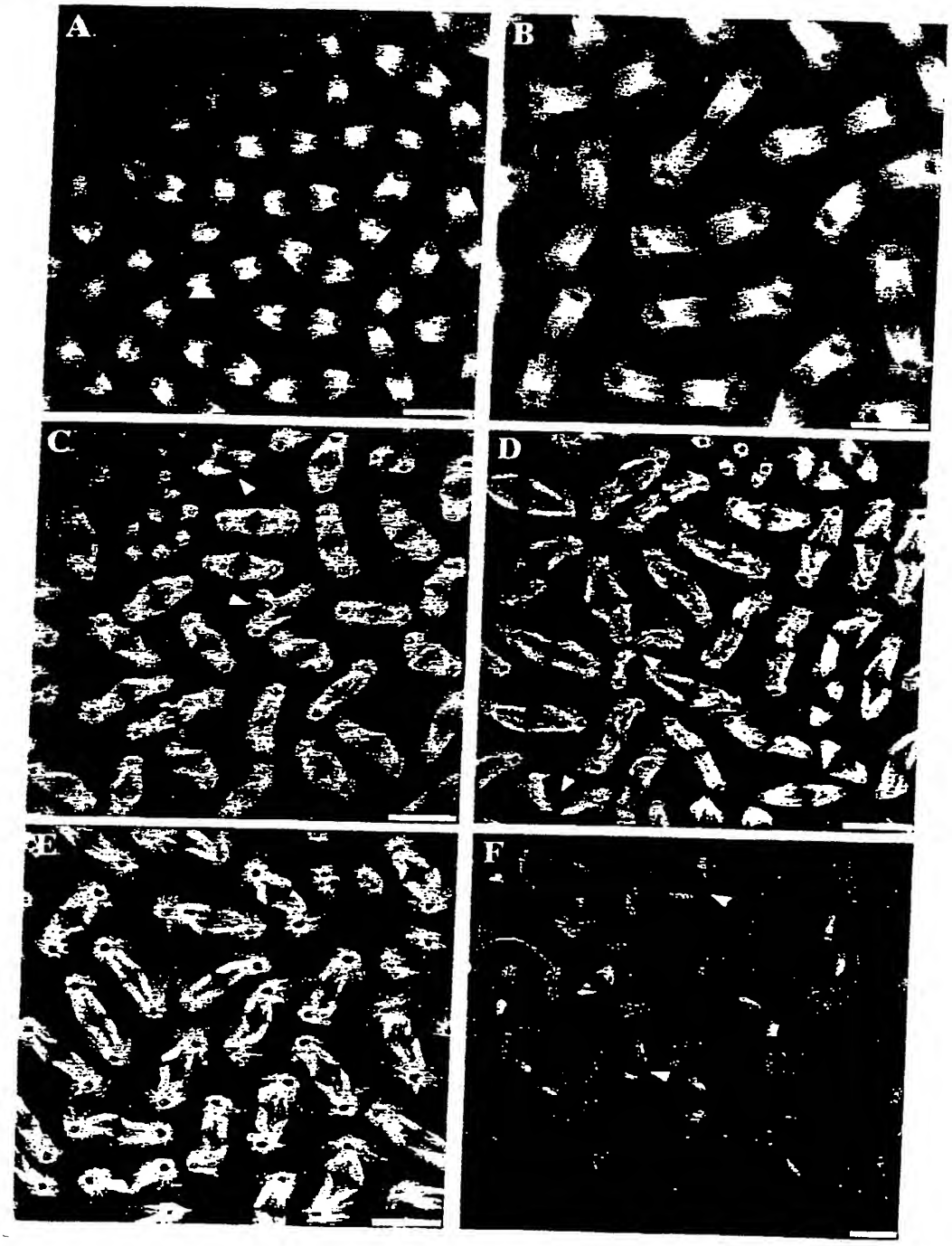


Figure 2

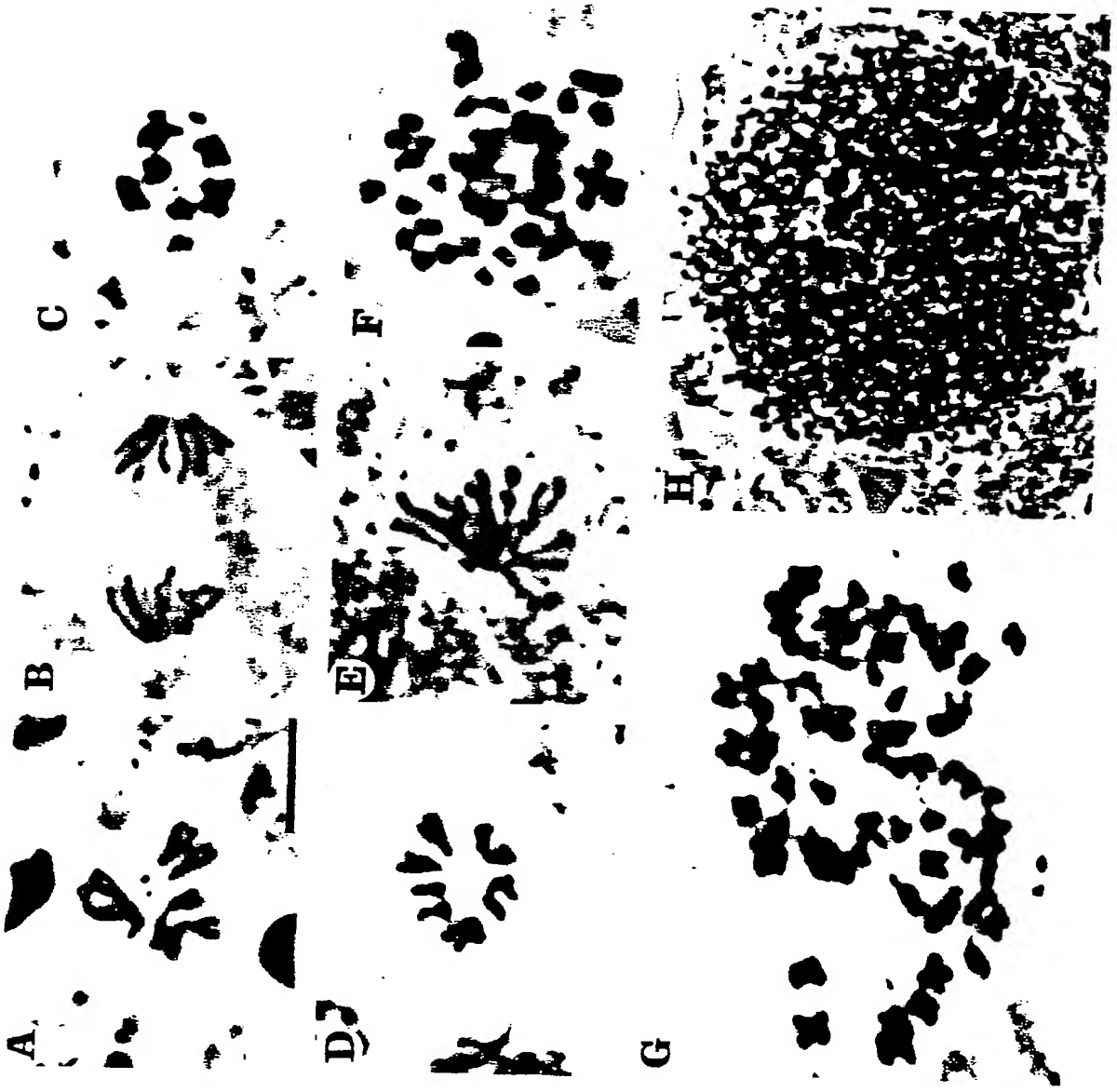


Figure 3

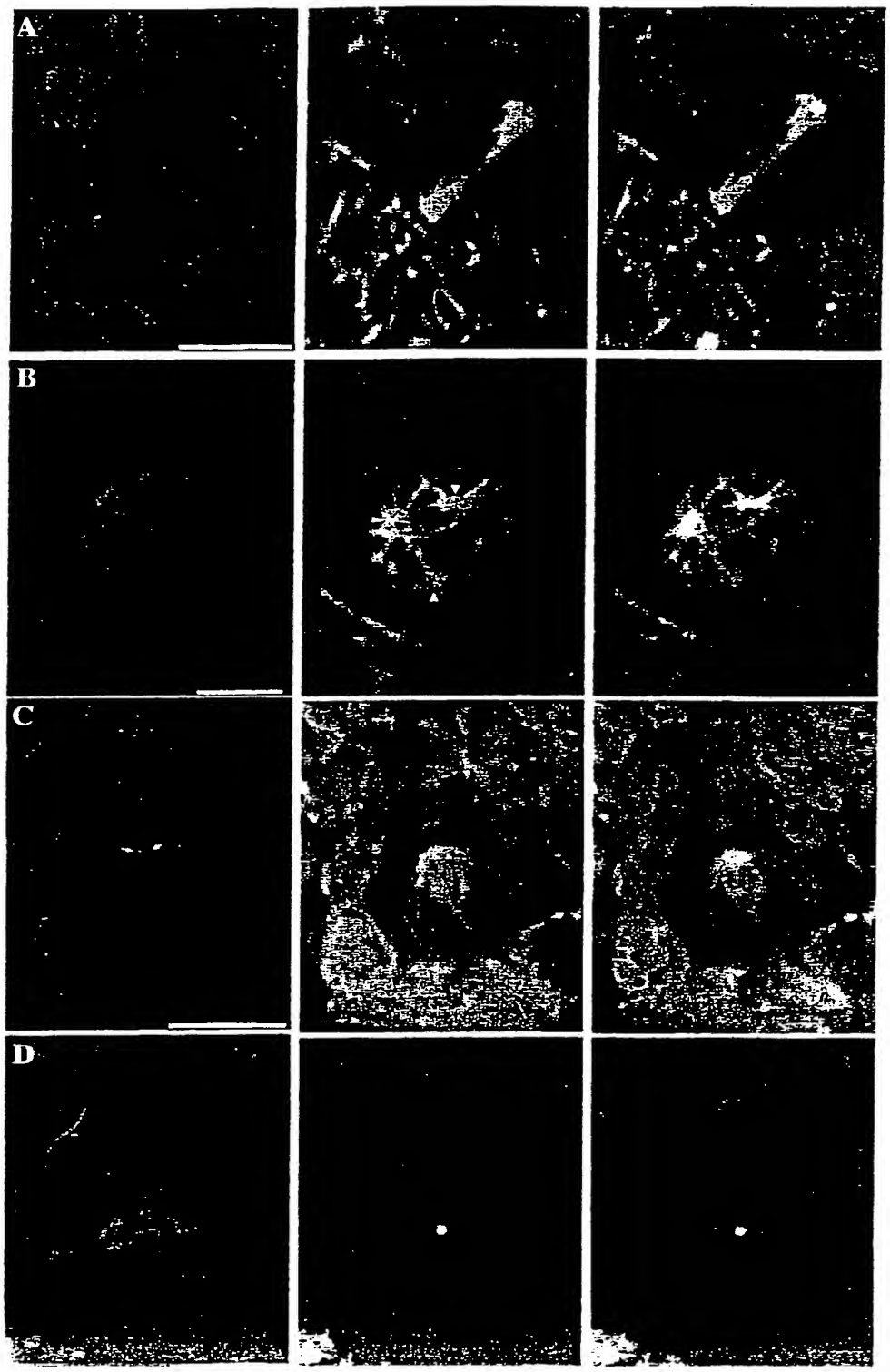


Figure 4

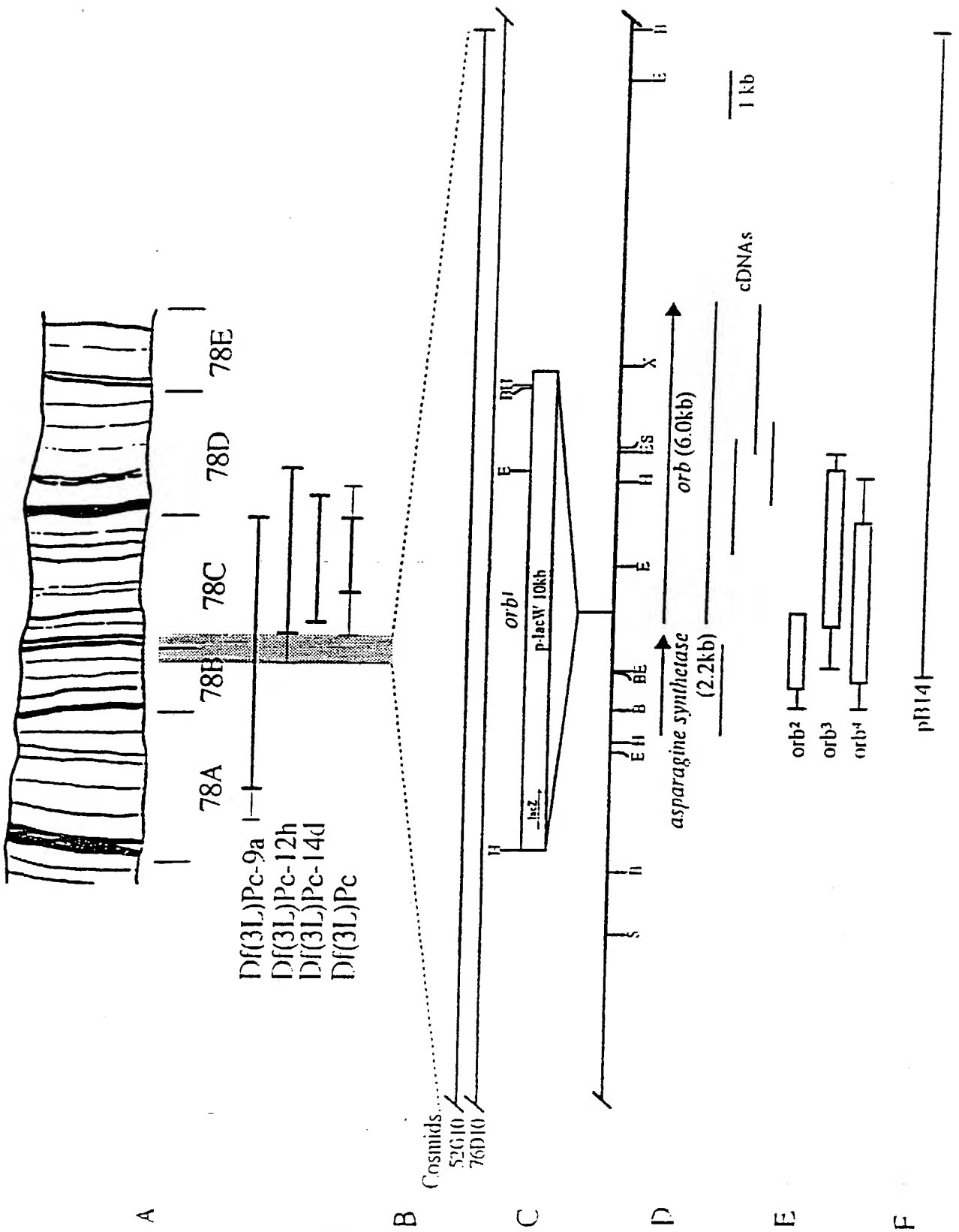


Figure 5

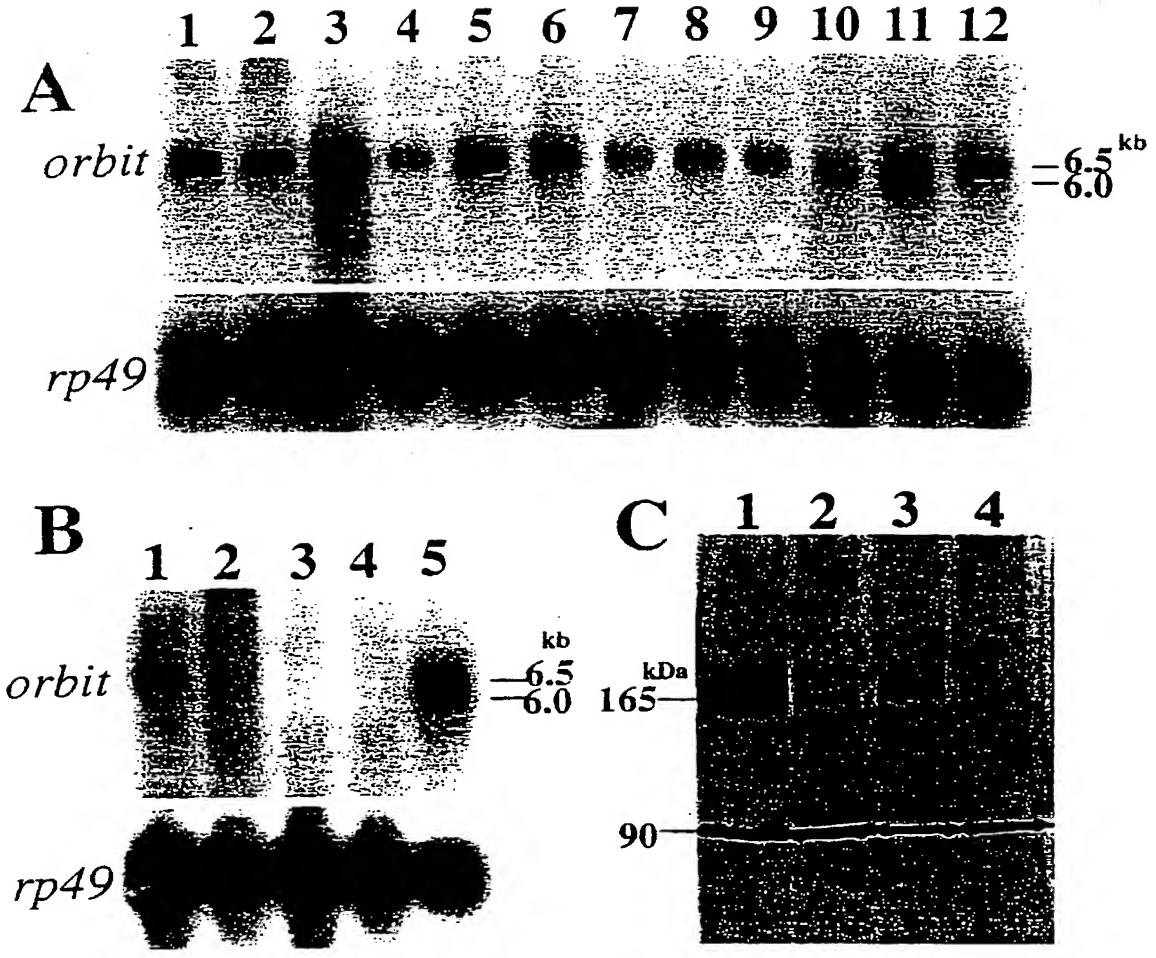
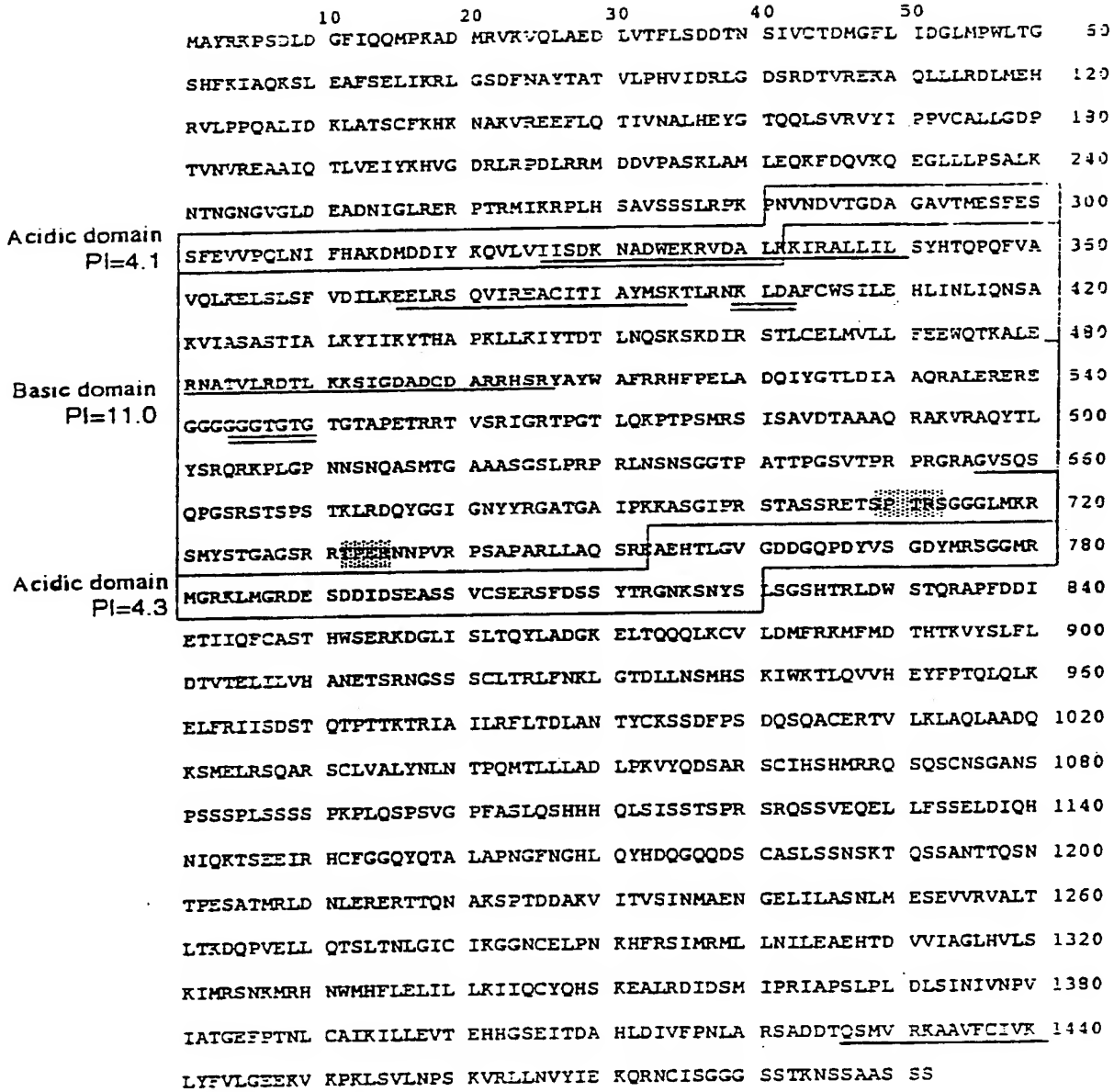

A
1 2 3 4 5 6 7 8 9 10 11 12
orbit
rp49
kb
6.5
6.0
B
1 2 3 4 5
orbit
rp49
kb
6.5
6.0
C
1 2 3 4
kDa
165
90

Figure 6



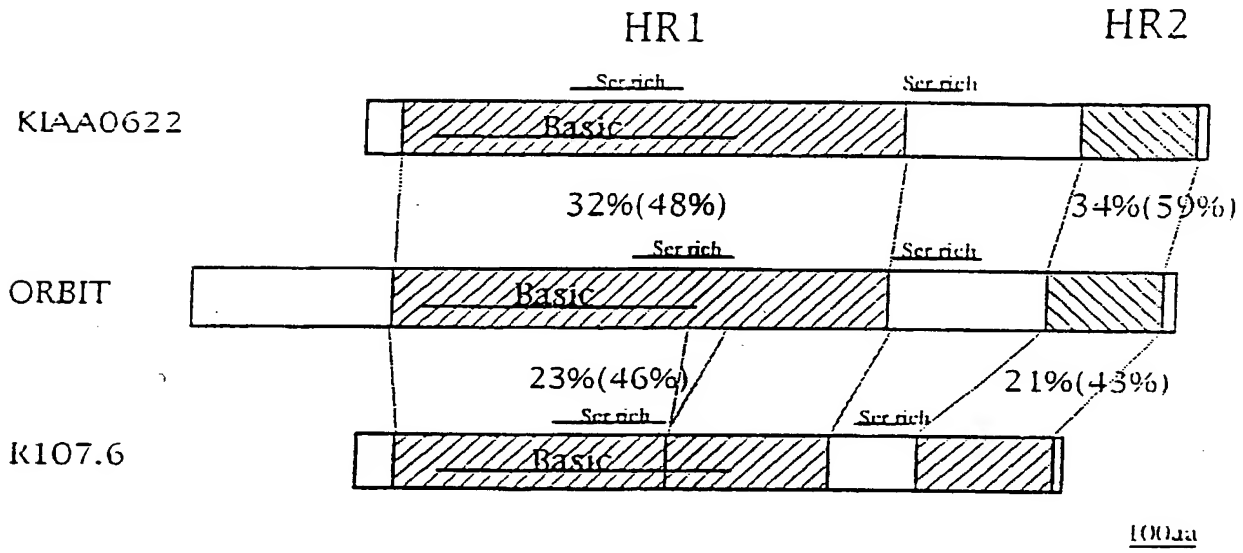
▒▒▒ P34^{cdc2} consensus phosphorylation sites

——— coserved sequences

═══ putative GTP-binding sites

Figure 7

A



B

Conserved Sequence Motifs in HR1

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KIAA0622        IISDDKHDWEQRVNALKKIRSLLLA
KIAA0627        IISDDKHDWDQRVNALKKIRSLLLA
R106.7          TISKGQEDWNKRMNKLKQIRSIVVH
ZC84.3          IISNSSEDWNKRQTQLKTVRSLVIH
MAP4            IVKDMELPTEKEVALVKDVRWPTET

                376
ORBIT           EELRSQVIREACITIAYMS
KIAA0622        KDLRSQVVREACITIGHIS
KIAA0627        KDLRSQVVREACITIAHIS
R106.7          KDLRSQIVREAAITCGFIF
ZC84.3          KDLRSQVIREAAITCSFIV

                479
ORBIT           LERNATVLRDTLKKSIGDADCDARRHSR
KIAA0622        LERHISVLAETIKKGIHDADSEARIEAR
KIAA0627        LERHAAVLVETIKKGIHDADAEARVEAR
R106.7          KRTVLEQIGELIKAAICDADPETRVAGR
ZC84.3          KKQIMRQICELIKSAINDADSETRAAGR
STU1            LENNIIYIEEWLKKGISDSQTTVREAMR

                654
ORBIT           RA-G-VSQSQPGSRSTSPS
KIAA0622        RAKV-VSQSQR-SRSANPA
KIAA0627        RTKM-VSQSQPGSRSGSPG
R106.7          RARGSVETSQPGSRNGSPE
```

Conserved Sequence Motif in HR2

```
                1426
ORBIT           TQSMVRKAAVFCIV
KIAA0622        TESSVRKASVFCIV
KIAA0627        SESSVRKACVFCIV
R106.7          TQSSVRKAAVFCIV
ZC84.3          TSSTVRKTVVYCIV
```

Figure 8

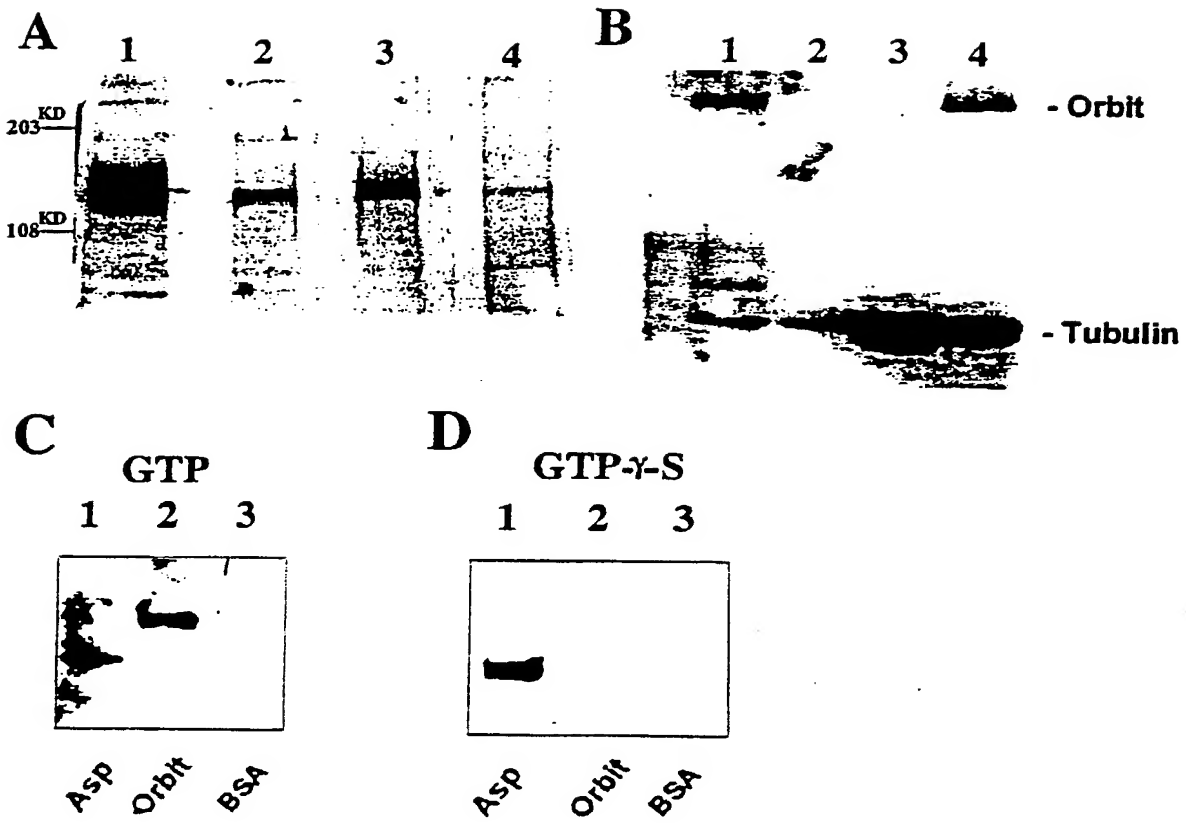
A
1
2
3
4
203 KD
108 KD
B
1
2
3
4
- Orbit
- Tubulin
C
GTP
1
2
3
Asp
Orbit
BSA
D
GTP-γ-S
1
2
3
Asp
Orbit
BSA

Figure 9

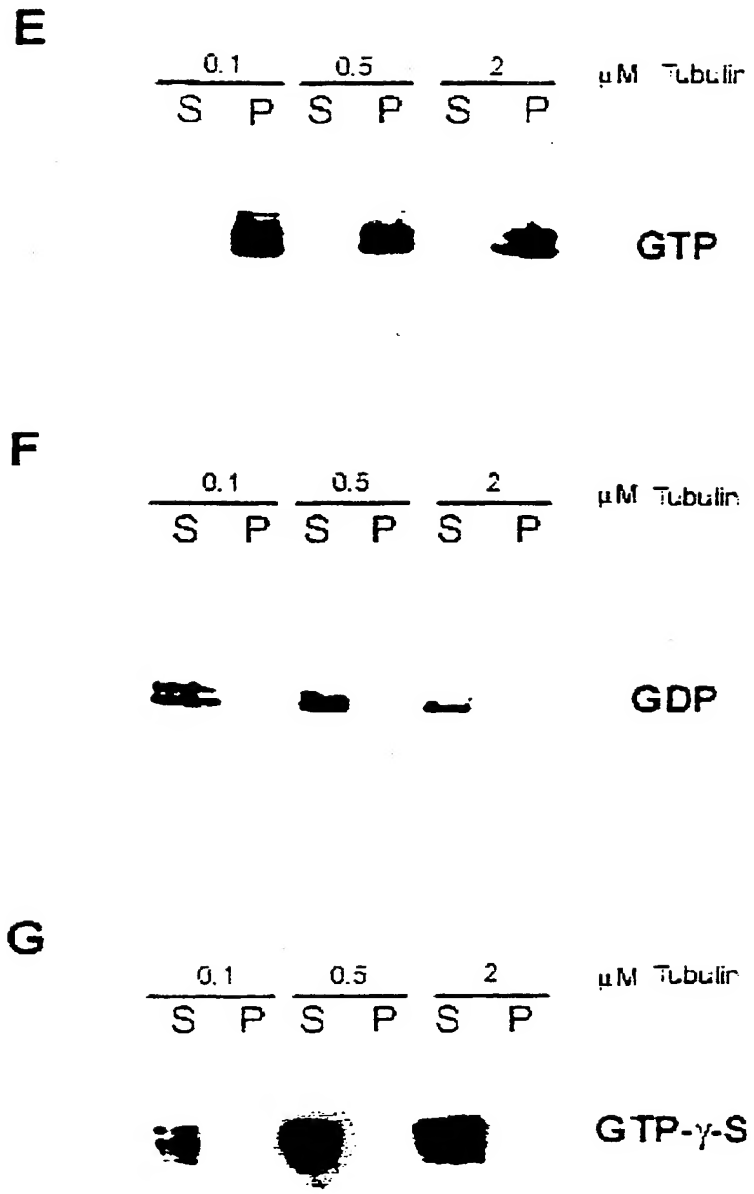

Figure 9 continued

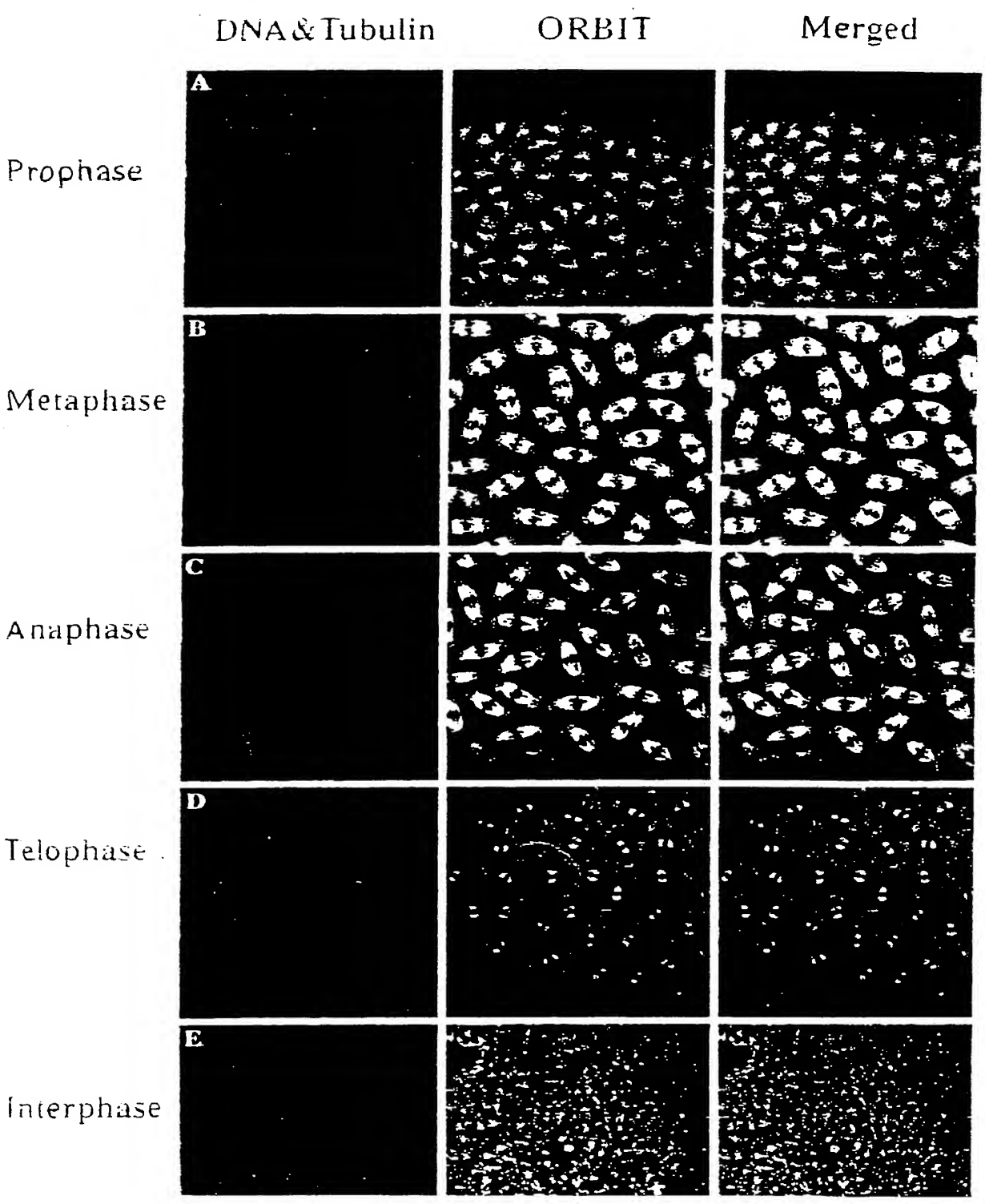
DNA & Tubulin
ORBIT
Merged
A
Prophase
B
Metaphase
C
Anaphase
D
Telophase
E
Interphase

Figure 10

DECLARATION, PETITION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(Check one):

☐ Declaration Submitted with Initial Filing

☒ Declaration Submitted after Initial Filing

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ORBIT AND HOMOLOGUES THEREOF

the specification of which (check one):

☐ is attached hereto.

OR

☒ was filed on **11 July 2000** as PCT International Application Number **PCT/GB00/02662** and filed as .

☐ and was amended by PCT Article 19 Amendment on _____ (if applicable),

☐ and was amended by PCT Article 34 Amendment on _____ (if applicable).

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

PRIORITY CLAIM

(Check one):

☐ no such applications have been filed.

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Prior Foreign Application Number(s)	Country	Foreign Filing Date (dd,mm,yyyy)	Priority Not Claimed	Certified Copy Attached Yes	Certified Copy Attached No
9916402.2	**GB**	**13 July 1999 (13.07.99)**	☐	☐	☒
9930707.6	**GB**	**24 December 1999 (24.12.99)**	☐	☐	☒

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Provisional Application Number(s)	Filing Date (dd/mm/yyyy)

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

3) **U.S./PCT PRIORITY CLAIM:** I hereby claim the benefit under Title 35, United States Code, §120 of any United States application or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (dd/mm/yyyy)	Parent Patent Number *(if applicable)*

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Ralph A. Loren	Reg. No. 29,325	Maria C. Laccotripe	Limited Recognition
Giulio A. DeConti, Jr.	Reg. No. 31,503		Under 37 C.F.R. § 10.9(b)
Ann Lamport Hammitte	Reg. No. 34,858	Debra J. Milasincic	Reg. No. 46,931
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Direct Telephone Calls to: (name and telephone number)

Giulio A. DeConti, Jr., (617) 227-7400

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor **Maria do Carmo AVIDES**
Inventor's signature Maria do Carmo Avides Date 10th October 2002
Residence **Moreira, 5 Causeway, Fen Causeway, Cambridge CB3 9EH, Great Britian** GBX
Citizenship **Portugal**
Post Office Address (if different)

Full name of sole or first inventor **Peter DEAK**
Inventor's signature Date
Residence **27 George Nuttal Close, Cambridge CB4 1YE, Great Britian**
Citizenship **Great Britian**
Post Office Address (if different)

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Inventor's signature Date
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Citizenship **Portugal**
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Full name of sole or first inventor **Peter DEAK**
Inventor's signature Peter Deak Date 5/8/2002
Residence **27 George Nuttal Close, Cambridge CB4 1YE, Great Britian** GBX
Citizenship ~~**Great Britian**~~ HUNGARIAN
Post Office Address (if different) FÖFASOR 138, H-6726 SZEGED, HUNGARY

2-00

Full name of sole or first inventor **David Moore GLOVER**
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Post Office Address (if different)

Full name of sole or first inventor **David Moore GLOVER**
Inventor's signature Date 27-5-02
Residence **Vincent Cottage, 20 Fox Street, Great Gransden, Sandy, Bedfordshire SG19 3AA** **Great Britian** GBX
Citizenship **Great Britian**
Post Office Address (if different)

SEQUENCE LISTING

SEQ ID NO. 1 - Orbit nucleotide sequence

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Glover, David M

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         35                  40                  45

Phe Leu Ile Asp Gly Leu Met Pro Trp Leu Thr Gly Ser His Phe Lys
     50                  55                  60

Ile Ala Gln Lys Ser Leu Glu Ala Phe Ser Glu Leu Ile Lys Arg Leu
 65                  70                  75                  80

Gly Ser Asp Phe Asn Ala Tyr Thr Ala Thr Val Leu Pro His Val Ile
                 85                  90                  95

Asp Arg Leu Gly Asp Ser Arg Asp Thr Val Arg Glu Lys Ala Gln Leu
            100                 105                 110

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145                 150                 155                 160

Thr Gln Gln Leu Ser Val Arg Val Tyr Ile Pro Pro Val Cys Ala Leu
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Leu Gly Asp Pro Thr Val Asn Val Arg Glu Ala Ala Ile Gln Thr Leu
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Val Glu Ile Tyr Lys His Val Gly Asp Arg Leu Arg Pro Asp Leu Arg
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Arg Met Asp Asp Val Pro Ala Ser Lys Leu Ala Met Leu Glu Gln Lys
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Asn Thr Asn Gly Asn Gly Val Gly Leu Asp Glu Ala Asp Asn Ile Gly
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Leu Arg Glu Arg Pro Thr Arg Met Ile Lys Arg Pro Leu His Ser Ala
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Asp Ala Gly Ala Val Thr Met Glu Ser Phe Glu Ser Ser Phe Glu Val
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Val Pro Gln Leu Asn Ile Phe His Ala Lys Asp Met Asp Asp Ile Tyr
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Lys Gln Val Leu Val Ile Ile Ser Asp Lys Asn Ala Asp Trp Glu Lys
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Arg Val Asp Ala Leu Lys Lys Ile Arg Ala Leu Leu Ile Leu Ser Tyr
            340                 345                 350

His Thr Gln Pro Gln Phe Val Ala Val Gln Leu Lys Glu Leu Ser Leu
        355                 360                 365

Ser Phe Val Asp Ile Leu Lys Glu Glu Leu Arg Ser Gln Val Ile Arg
    370                 375                 380

Glu Ala Cys Ile Thr Ile Ala Tyr Met Ser Lys Thr Leu Arg Asn Lys
385                 390                 395                 400

Leu Asp Ala Phe Cys Trp Ser Ile Leu Glu His Leu Ile Asn Leu Ile
                405                 410                 415

Gln Asn Ser Ala Lys Val Ile Ala Ser Ala Ser Thr Ile Ala Leu Lys
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Tyr Ile Ile Lys Tyr Thr His Ala Pro Lys Leu Leu Lys Ile Tyr Thr
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Asp Thr Leu Asn Gln Ser Lys Ser Lys Asp Ile Arg Ser Thr Leu Cys
    450                 455                 460

Glu Leu Met Val Leu Leu Phe Glu Glu Trp Gln Thr Lys Ala Leu Glu
465                 470                 475                 480

Arg Asn Ala Thr Val Leu Arg Asp Thr Leu Lys Lys Ser Ile Gly Asp
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Ala Asp Cys Asp Ala Arg Arg His Ser Arg Tyr Ala Tyr Trp Ala Phe
            500                 505                 510

Arg Arg His Phe Pro Glu Leu Ala Asp Gln Ile Tyr Gly Thr Leu Asp
        515                 520                 525

Ile Ala Ala Gln Arg Ala Leu Glu Arg Glu Arg Glu Gly Gly Gly Gly
    530                 535                 540

Gly Gly Thr Gly Thr Gly Thr Gly Thr Ala Pro Glu Thr Arg Arg Thr
545                 550                 555                 560

Val Ser Arg Ile Gly Arg Thr Pro Gly Thr Leu Gln Lys Pro Thr Pro
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Ser Met Arg Ser Ile Ser Ala Val Asp Thr Ala Ala Ala Gln Arg Ala
            580                 585                 590

Lys Val Arg Ala Gln Tyr Thr Leu Tyr Ser Arg Gln Arg Lys Pro Leu
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Gly Pro Asn Asn Ser Asn Gln Ala Ser Met Thr Gly Ala Ala Ala Ser
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Gly Ser Leu Pro Arg Pro Arg Leu Asn Ser Asn Ser Gly Gly Thr Pro
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Gly Ala Ile Pro Lys Lys Ala Ser Gly Ile Pro Arg Ser Thr Ala Ser
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725 730 735

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740 745 750

Glu Ala Glu His Thr Leu Gly Val Gly Asp Asp Gly Gln Pro Asp Tyr
755 760 765

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770 775 780

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785 790 795 800

Val Cys Ser Glu Arg Ser Phe Asp Ser Ser Tyr Thr Arg Gly Asn Lys
805 810 815

Ser Asn Tyr Ser Leu Ser Gly Ser His Thr Arg Leu Asp Trp Ser Thr
820 825 830

Gln Arg Ala Pro Phe Asp Asp Ile Glu Thr Ile Ile Gln Phe Cys Ala
835 840 845

Ser Thr His Trp Ser Glu Arg Lys Asp Gly Leu Ile Ser Leu Thr Gln
850 855 860

Tyr Leu Ala Asp Gly Lys Glu Leu Thr Gln Gln Gln Leu Lys Cys Val
865 870 875 880

Leu Asp Met Phe Arg Lys Met Phe Met Asp Thr His Thr Lys Val Tyr
885 890 895

Ser Leu Phe Leu Asp Thr Val Thr Glu Leu Ile Leu Val His Ala Asn
900 905 910

Glu Thr Ser Arg Asn Gly Ser Ser Ser Cys Leu Thr Arg Leu Phe Asn
915 920 925

Lys Leu Gly Thr Asp Leu Leu Asn Ser Met His Ser Lys Ile Trp Lys
930 935 940

Thr Leu Gln Val Val His Glu Tyr Phe Pro Thr Gln Leu Gln Leu Lys
945 950 955 960

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Thr Arg Ile Ala Ile Leu Arg Phe Leu Thr Asp Leu Ala Asn Thr Tyr
            980                 985                 990

Cys Lys Ser Ser Asp Phe Pro Ser Asp Gln Ser Gln Ala Cys Glu Arg
        995                1000                1005

Thr Val Leu Lys Leu Ala Gln Leu Ala Ala Asp Gln Lys Ser Met Glu
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Asp Ser Ala Arg Ser Cys Ile His Ser His Met Arg Arg Gln Ser Gln
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Ser Cys Asn Ser Gly Ala Asn Ser Pro Ser Ser Ser Pro Leu Ser Ser
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Ser Ser Pro Lys Pro Leu Gln Ser Pro Ser Val Gly Pro Phe Ala Ser
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Asp Ile Gln His Asn Ile Gln Lys Thr Ser Glu Glu Ile Arg His Cys
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Phe Gly Gly Gln Tyr Gln Thr Ala Leu Ala Pro Asn Gly Phe Asn Gly
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His Leu Gln Tyr His Asp Gln Gly Gln Gln Asp Ser Cys Ala Ser Leu
   1170                1175                1180

Ser Ser Asn Ser Lys Thr Gln Ser Ser Ala Asn Thr Thr Gln Ser Asn
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Thr Pro Glu Ser Ala Thr Met Arg Leu Asp Asn Leu Glu Arg Glu Arg
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Thr Thr Gln Asn Ala Lys Ser Pro Thr Asp Asp Ala Lys Val Ile Thr
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Val Ser Ile Asn Met Ala Glu Asn Gly Glu Leu Ile Leu Ala Ser Asn
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Gln Pro Val Glu Leu Leu Gln Thr Ser Leu Thr Asn Leu Gly Ile Cys
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Ile Ala Gly Leu His Val Leu Ser Lys Ile Met Arg Ser Asn Lys Met
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Arg His Asn Trp Met His Phe Leu Glu Leu Ile Leu Leu Lys Ile Ile
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Gln Cys Tyr Gln His Ser Lys Glu Ala Leu Arg Asp Ile Asp Ser Met
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Val Asn Pro Val Ile Ala Thr Gly Glu Phe Pro Thr Asn Leu Cys Ala
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Pro Gly

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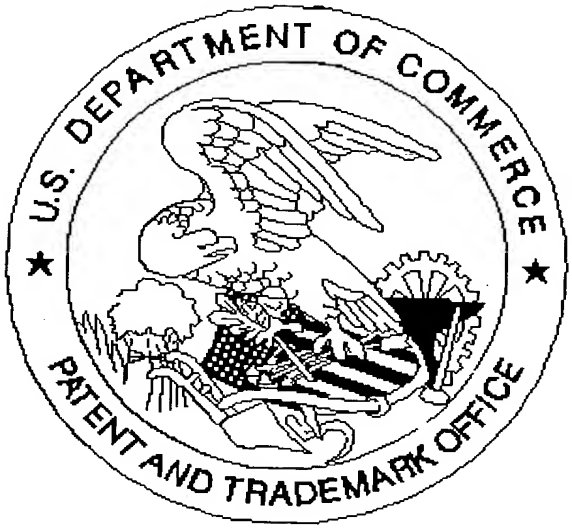

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